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Introduction

A major challenge for treating prostate cancer (PC) is to discover new therapies that will prevent the spread of PC cells from the prostate to distal sites. Our research focuses on the stretch-activated mechanosensitive Ca^{2+} permeant channel (MscCa) as a central regulator of prostate tumor cell migration. Our experiments are designed to address the two most basic issues of the disease: the mechanism(s) that trigger progression of PC to malignancy and the urgent need for new therapeutic targets to block or reverse this progression. Our original experiments funded by DOD were aimed to test whether MscCa is expressed in human prostate tumor cells and whether MscCa activity is required for prostate tumor cell migration. We confirmed both results. In the course of these experiments we also discovered that the predominate gating mode of the MscCa differs between noninvasive and invasive PC cells, may be the most powerful determinate of the $[Ca^{2+}]_i$ dynamics required to coordinate cell locomotion. The aims of the current award were three-fold. First, determine the mechanisms underlying MscCa gating. Second, determine the cancer-related processes that switch MscCa gating, and third determine whether anti-MscCa conditions that suppress PC migration in vitro also block PC cell invasion in vivo. Insights into these aspects would provide added motivation for developing more selective therapies that target MscCa and its regulatory mechanisms. The basic results supporting our hypothesis have been published (Maroto, R. & Hamill, O.P. MscCa regulation of tumor cell migration and metastasis. *Current Topics in Membranes*.59, 485-509, 2007). Also included in the Appendix are other manuscripts and abstracts (Hamill & Maroto 2007; 2008; Gotlieb et al., 2008; Maroto & Hamill, 2011; and Maroto, Kurosky and Hamill, 2011) that include specific results described in body of the text.

Body

In year 4, a no-cost 1 year extension, we readdressed Task 1 in order to identify the relation between MscCa and TRPCs in regulating PC cell migration (Maroto & Hamill 2007). Our basic results on both MscCa and TRPC1 on cell migration have been confirmed by other groups studying different migratory cell types (Fabian et al., 2008; Louis et al., 2008). The consensus is that agents that block MscCa (Gd^{3+} and GsmTx-4) and genetic treatments that silence TRPC1 inhibit cell migration. However, our new results also indicate that when TRPC1 is over expressed in mammalian cells it does not traffic to the plasma membrane nor does it result in increased MscCa expression (Gottlieb et al., 2008, see Appendix). In year 4, we reexamined the role of other TRPCs as known binding partners of TRPC1 to examine whether they can increase MscCa. We also tested whether another protein, TRPM7, may form MscCa and regulate PC cell migration (Maroto & Hamill, 2007; Wei et al., 2009; see also Hamill & Maroto, 2010, Appendix). The key findings of this year's work based on suppression and overexpression of TRPCs is that TRPC1 does not directly form MscCa in PC cells even though TRPC1 and TRPC3 expression (but not TRPC6) are required for PC cell migration (Maroto et al., 2011, Appendix). Furthermore, we have been unable to demonstrate TRPM7 expression in PC cell lines or detect TRPM7 related Ca2+ flickers in the lamelleipodium of migratory PC cells. We conclude that MscCa and specific TRPCs represent promising targets to block tumor cell migration. However, the protein identity of MscCa in human PC cells now needs to be reevaluated in relation to the recent report that members of a completely novel protein family (Piezo) form MscCa (Coste et al , 2010).

Task 1: Determine the mechanism(s) that regulate MscCa gating, expression and surface distribution in PC cells that display different invasiveness and metastatic potential.

1.1 Use patch-clamp/pressure clamp techniques, confocal immunofluorescence, Westerns and surface biotinylation techniques to measure gating, surface distribution and expression, respectively, of MscCa/TRPC1 in PC-3 and LNCaP cell lines.

Time line: Year 1, months 1-6.

Milestone: Establish a baseline for studying the effects of various agents and treatments that may alter these properties as described in tasks 1.2 to 1.4.

Task 1 has been completed and we show that MscCa is uniformly expressed over the cell surface of PC-3 with no evidence of surface concentration of MscCa at the front versus the rear (Fig.1). MscCa is more highly expressed in LNCaP versus PC-3 as is TRPC1 (measured by Westerns). Confocal immunofluorescence indicates that endogenous TRPC1 is expressed in a punctuate pattern in the rear tether of the PC-3 inconsistent with the uniform distribution of MscCa (Fig. 2). Overexpression of GFP-TRPC1 in mammalian cells indicates it is not trafficked to the membrane but tends to accumulate intracellularly (Maroto & Hamill, 2007, Gottlieb et al., 2008; Maroto et al., 2011a, b, see Appendix).

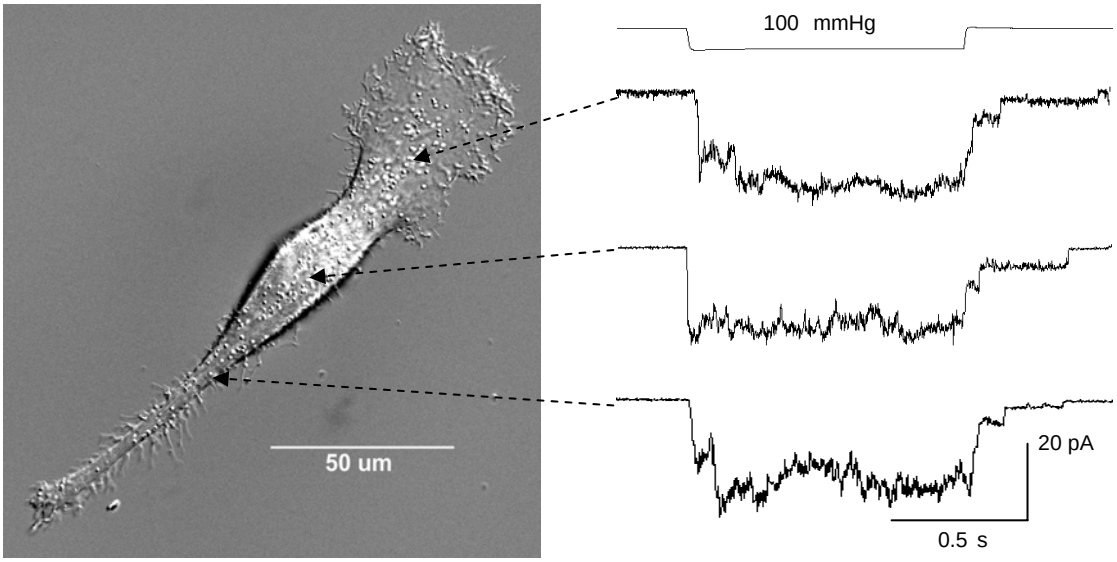

Figure 1. Cell-attached patch clamp recording from different regions of a migrating PC-3 cell indicates that MscCa is uniformly expressed over the membrane surface. The individual current responses to a 100 mmHg suction pulse were recorded form the indicated regions of different PC-3 cells.

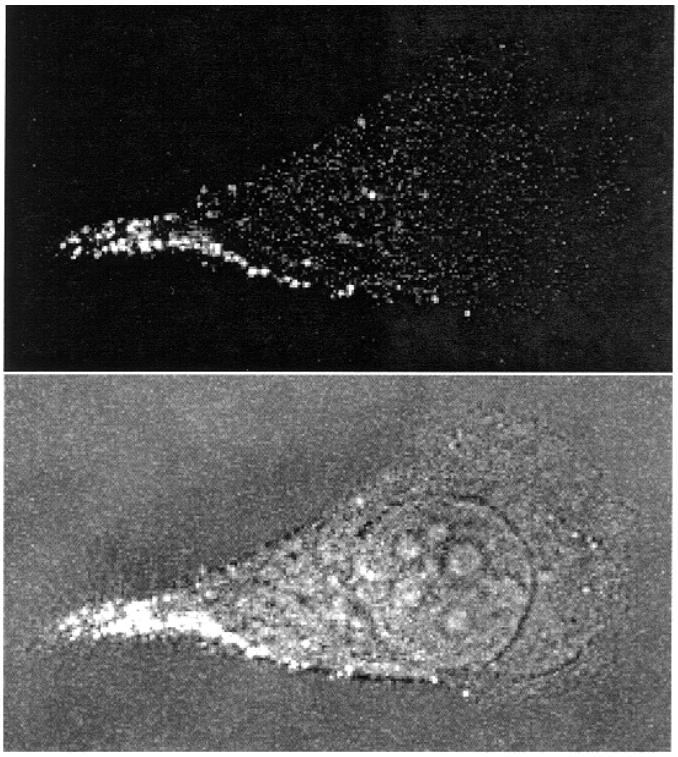

Figure 2. Confocal immunofluorescence microscopy indicates that TRPC1 is more highly expressed in a punctuate pattern at the trailing tether of a migrating PC-3 cell. This result indicates that TRPC1 may not form the uniformly expressed MscCa.

1.2 Use Westerns to establish the TRPCs (TRPC1-7) expressed in PC-3 and LNCaP cells. Use cDNA or short hair pin RNAs inserted in plasmid vectors in order to generate permanent PC-3 and LNCaP cell lines in which specific TRPCs have been either over expressed or silenced. Use methods of 1.1 to establish the functional properties of MscCa and how TRPC1 expression and surface distribution are alter. Use time-lapse $[Ca^{2+}]$i imaging to study $[Ca^{2+}]_i$ dynamics and migration in the various PC cell sub-lines.

Time line: Year 1, months 1-12.

Milestone: Role of specific TRPCs in determining PC cell specific MscCa properties and their influence on $[Ca^{2+}]_i$ dynamics and cell migration.

Task 1.2 is completed. Fig. 3 show Western blot measurements of expression of TRPC1, 3, 4, 5 and 6 in the highly motile PC-3 cells and weakly motile LNCaP cells. TRPC1 is more highly expressed in LNCaP cells, consistent with the higher density of MscCa in LNCaP; TRPC3 is also more highly expressed in LNCaP; TRPC4, although present in LNCaP cells was not detectable in PC-3 cells; TRPC5 was not detectable in either cell line even though the antibody detected the protein in Xenopus oocytes; TRPC6 was equally but only weakly expressed in PC-3 and LNCaP cells. TRPC2, a pseudogene in humans, and TRPC7 for which there were no commercially available antibodies were not studied here.

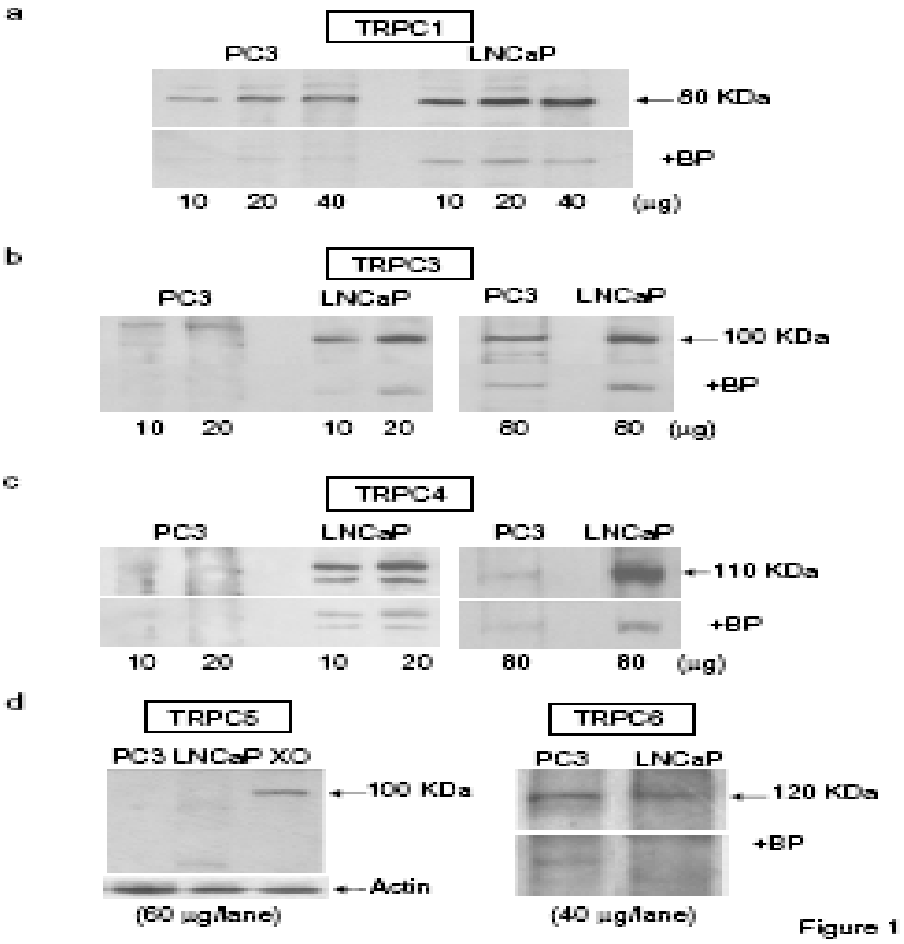

Figure 3. Westerns showing TRPC1, 3, 4, 5 and 6 expression in PC-3 and LNCaP cells lines. *Xenopus* oocytes (XO) were used as a positive control for the absence of TRPC5 in PC cells.

These results are significant because in addition to TRPC1 (Maroto et al., 2005) there are reports that TRPC5 and TRPC6 may form MscCa. However, since TRPC5 was not detected in PC cells and overexpression of TRPC6 does not increase endogenous MscCa activity we can rule out a direct involvement in MscCa (Gotlieb et al., 2008; Appendix).

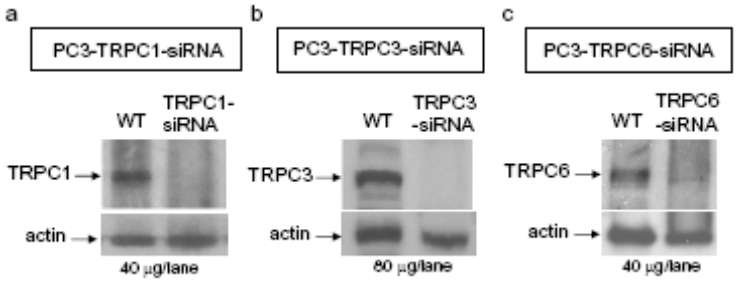

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Figure 4. Western blots comparing TRPC1, TRPC3 and TRPC6 expression in wild type PC-3 cells and in PC-3 sublines that were transfectecd with short hairpin RNAs to selectively and permanently suppress TRPC1, TRPC3 or TRPC6.

As reported in last year's annual report, we measured a ~50 % reduction in MscCa currents in TRPC1-suppressed PC cells compared with wild type PC controls (30 ± 5 pA vs 75 ± 8 pA based on ≥ 30 cells in each condition). However, this year we regenerated a permanent PC subline transfected with scrambled siRNA and observed a similar reduction in MscCa current when compared with the wild type PC-3 cell control (34 ± 4 pA, n =35). Similarly no difference in MscCa expression was observed with suppression of TRPC3 or TRPC6 when compared the scrambled control. These new results indicate off-target effects of siRNA transfection on MscCa expression that is independent of of specific TRPC suppression (Maroto et al., 2011; Appendix). Furthermore, as reported last year we have compared MscCa expression in PC cell sublines that permanently over express TRPC1 and TRPC3 as judged by the Westerns shown in Figure 5 and observed no significant change in MscCa current amplitude compared with either wild type PC cells or a PC cell subline over expressing TRPC6 (Maroto et al., 2011; Appendix).

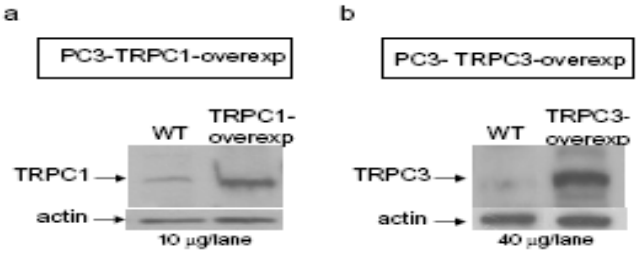

Figure 5. Western blots comparing TRPC1 and TRPC3 protein levels in wild type and in PC-3 sublines permanently transfected with TRPC1 or TRPC3.

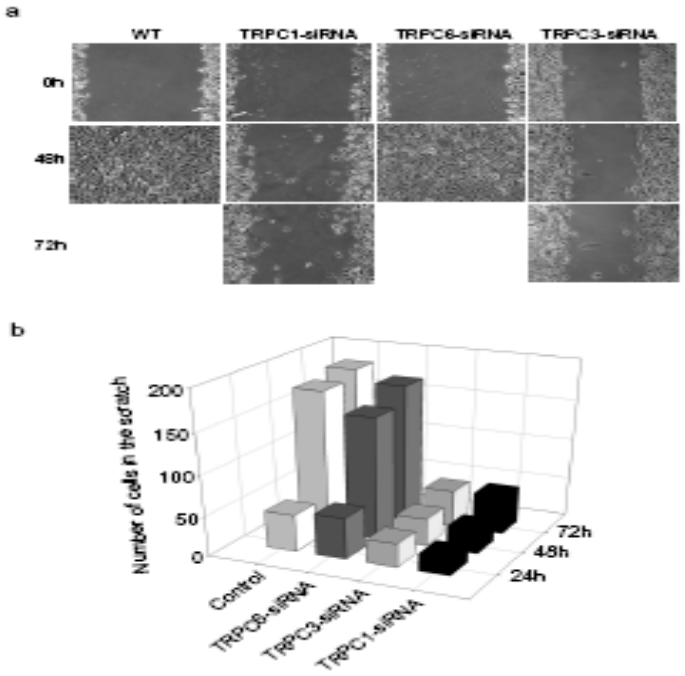

Figure 6. Wound/scratch closure assays used to measure the migratory function of wild type PC-3 cells and PC-3 sublines in which TRPC1, 3 or 6 were permanently suppressed.

Despite the lack of specific effects of TRPC1/TRPC3 suppression on MscCa activity, PC-3 cell migration as measured by wound-closure assay as well time-lapse video microscopy results (Fig. 6, see also Fabian et al., 2008; Louis et al., 2008). In this case the control used for off-target effects of siRNA transfection and nonspecific TRPC suppression was a PC-3 subline in which TRPC6 was suppressed but showed similar and complete wound closure as displayed by wild type PC-3 cells (Fig. 6).

Tasks 1.1 and 1.2 has been completed in years 1 and 2 and the results form the manuscripts (Maroto & Hamill, 2007, Maroto, Kurosky & Hamill, 2011, Appendix).

Deliverables: We have generated permanent PC-3 cells lines in which TRPC1 and 3 were suppressed or over expressed and another PC-3 cell line in which TRPC6 was suppressed.

The conclusion from Tasks 1.2 is that although TRPC 1 & 3 expression is required for PC cell migration, they do not act via MscCa. This result is inconsistent with our original hypothesis (Maroto & Hamill 2007, Hamill & Maroto, 2007; Maroto et al., 2011, Appendix) and has stimulated additional experiments in years 3 and 4 that were not part of our original proposal but are key for understanding the molecular basis of MscCa block of PC-3 cell migration as well as completing parts of Tasks 2 & 3. In particular, we had hypothesized that another TRP family member, TRPM7, because of its reported stretch sensitivity and expression at the leading edge of migratory cells, may also participate in regulating PC tumor cell migration (Maroto & Hamill, 2007). Indeed rapid confocal measurements of $[Ca^{2+}]_i$ changes in the lamellipodia of migrating fibroblasts have revealed Ca2+ transients ("Ca2+ flickers") that were required for both directional migration and chemotaxis, and were reported to depend upon TRPM7 expression (Wei et al., 2009; see also Hamill & Maroto, 2009 included in appendix).

During 2009/2010 we attempted to confirm a role for TRPM7 in forming MscCa and also record Ca2+ flickers in PC cells. However, in both cases we were unsuccessful and despite extensive trouble shooting of these two issues. First, we were unable to detect TRPM7 in PC cells in Westerns using the same antibody used by Wei et al., 2009 (Fig. 7A). Similarly, we did not detect TRPM7 using a monoclonal anti-TRPM7 antibody generated by Abcam. These negative results were puzzling because we tested several different batches of antibodies did not detect the predicted 200 kDa band in the positive control (GH3 cells and rat brains) indicated in the manufacturer's data sheet (Figure 7B). We have contacted the companies and several colleagues, who informed us that TRPM7 antibodies in general suffer from non-specificity. This was supported by our observation that the antibodies detected a ~90 kDa band in PC3, LNCaP, GH3, and rat brain. However, labeling of the band was not blocked by preincubation with the control peptides (compare figure 7B). Sunsequently, we were informed by Abcam that there monoclonal antibody is used to detect over expressed TRPM7 rather than endogenous TRPM7. Despite this we have generate a TRPM7 suppressed PC cell line. However, patch clamp recordings indicate that MscCa is not blocked in this subline compared to the scrambled control. This negative result regarding TRPM7 involvement in MscCa is consistent with reports that TRPM7 channels different properties (outward rectification) compared with MscCa (inward rectification).

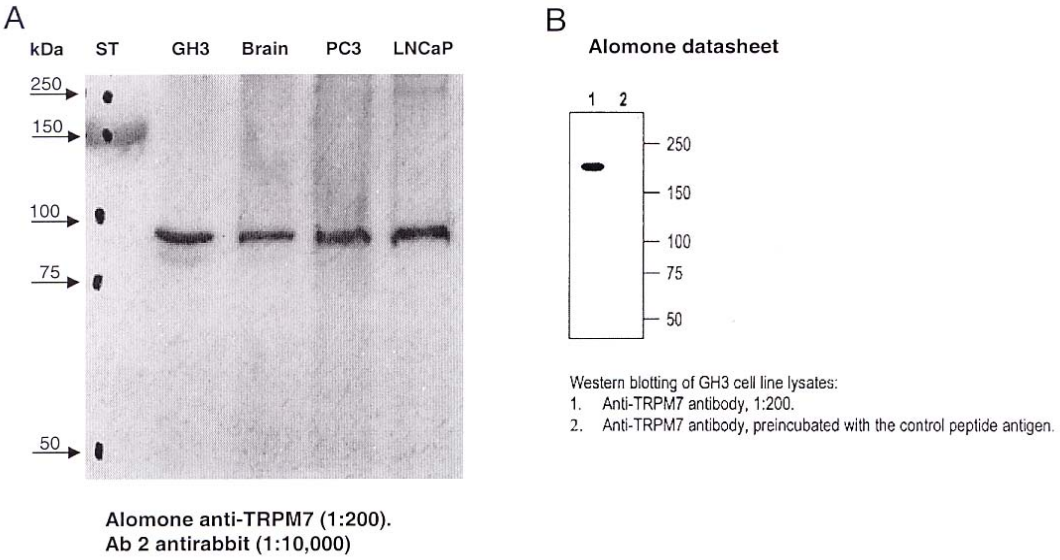

Figure 7. Western blots using a polyclonal anti-TRPM7 antibody (Alomone) to measure proteins in GH3, rat brain PC-3 and LNCaP cells. A: shows labeling a a 90 kDa band thatwas not specific because it was not blocked by the control peptide (not shown). B: Alomone data sheet showing labeling of a ~200 KDa band in GH-3 cells that was blocked by the control peptide.

PC-3 cells show particularly pronounced lamelleipodia (see Figure 1) and therefore should be amenable to detecting the Ca2+ flickers reported by Wei et al., 2009. However, employing the same model of Zeiss confocal microscope, the same imaging protocol, and the same culture conditions used by Wei et al., 2009 we have been unable to detect Ca^{2+} flickers in migrating PC-3 cells. We have contacted the senior authors (C. Wei and H. Cheng) of the paper for specific recommendations and even studied their same fibroblast cell line but again detected no flickers. We are continuing these measurements with the UTMB optical facility. At least according to the most recent Pubmed no other group has confirmed either the Ca2+ flickers or the role of TRPM7 in forming MscCa in migrating cells. As mentioned in the introduction a recent report indicates that members of a completely novel protein family (Piezo) form MscCa (Coste et al , 2010) and display the same single channel properties we record in PC cells (i.e., similar conductance and inward rectification)

1.3 Use specific agents that either promote actin depolymerization (Latrunculin A) or polymerization (jasplakinolide) to study the effects on MscCa properties and $[Ca^{2+}]_i$ dynamics and cell motility on PC cell lines.

Time line: Year 1, months 9-12; Year 2, months 1-6.

Milestone: The role of the actin-CSK in regulating MscCa properties.

1.4 Use treatments (methyl-β-cyclodextrin with/without cholesterol) in order to deplete or enrich the bilayer with cholesterol and measure the effects on MscCa properties.

Time line: Year 2 months 6-12.

Milestone: The role of lipid bilayer structure in determining MscCa properties and PC cell motility.

Deliverables: Agents that regulate MscCa properties and thereby the motility of PC cells.

Our results indicate that the different MscC gating modes seen in LNCaP and PC-3 cells should be strong determinants of the mechanical induced Ca2+ influx into PC cells. Our results also indicate that the transient gating of MscCa of LNCaP cells is highly sensitive to specific forms of mechanical disruption. In particular, whereas repetitive 100 ms suction pulses have no effect on the transient gating mode, the application of 1 second pulses causes a progressive and irreversible conversion of the transient gating mode to the sustained gating mode that is more PC-3 cell-like (Maroto & Hamill, 2007, Maroto et al., 2011a,b see Appendix). Based on this mechanical fragility we proposed that sustained mechanical stimuli may act by disrupting the actin cytoskeleton directly underlying the membrane. However, our experiments do not support our original hypothesis, using cytochalasin D (Fig. 8) and latrunculin at concentrations (5-10 micromolar) and incubation times (> 1 hour) known to cause extensive actin deplolymerization left the transient gating mode intact. Similarly, jasplakinolide (200 nM for 2 hours), an agent reported to promote actin polymerization also did not alter MscCa transient gating in LNCaP cells. However, curcumin (60 micromolar for 30 minutes) which also promotes actin polymerization reduced the transient gating mode. This result did not fit with our predictions and does not fit with lack of effect of jasplakinolide.

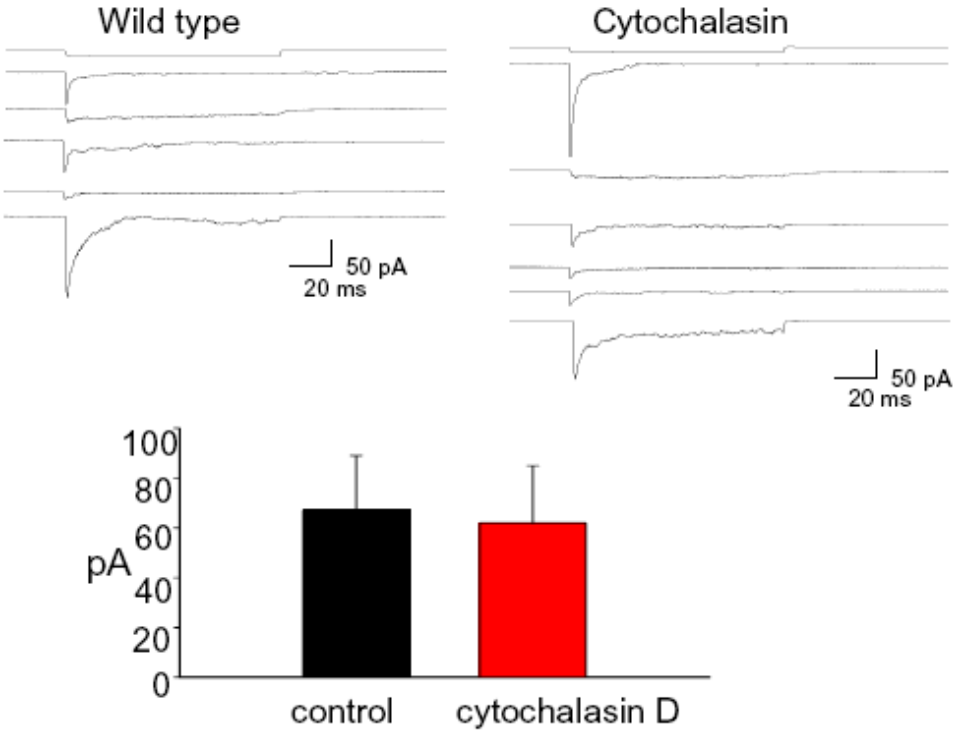

Figure 8. Cytochalasin treatment (10 μM for 4 hours) which is know to causes significant actin depolymerization but does not affect the peak amplitude or fast inactivation of MscCa recorded in LNCaP cells. The current traces are from 5 control cells and 6 cytochalasin treated cells.

We find that Methyl-β-cyclodextrin at concentrations and incubations times (10 mM and 60 minutes) that are know to deplete membrane cholesterol do not switch the transient MscCa gating mode, indicating that changes in membrane lipid fluidity do regulate rapid MscCa gating/inactivation. Our new studies carried this year indicate that the different intrinsic mechanical properties of PC-3 and LNCaP cells, which are reflected in the different suction pressure protocols required in forming tight seals on the PC cells, may be the strongetst determinant whether one records transient or sustained gating modes during cell-attached patch recordings. In particular, because stronger and more prolonged suctions are required to form seals on PC-3 cells compared with LNCaP cells, and mechanical stimulation irreversibly converts MscCa gating from the transient to the sustained gating mode in LNCaP cells, the sustained gating mode seen in PC-3 cells may be a reflection of the different sealing protocol rather than PC cell specific differences in regulation of MscCa gating (i.e., our original hypothesis). In this case, alternative methods are required to measure MscCa gating in PC cells that are independent of the tight cell patch recording technique. One possibility we are considering is the use of a piezoelectric controlled probe to directly compress the PC cell while recording in the whole cell recording mode.

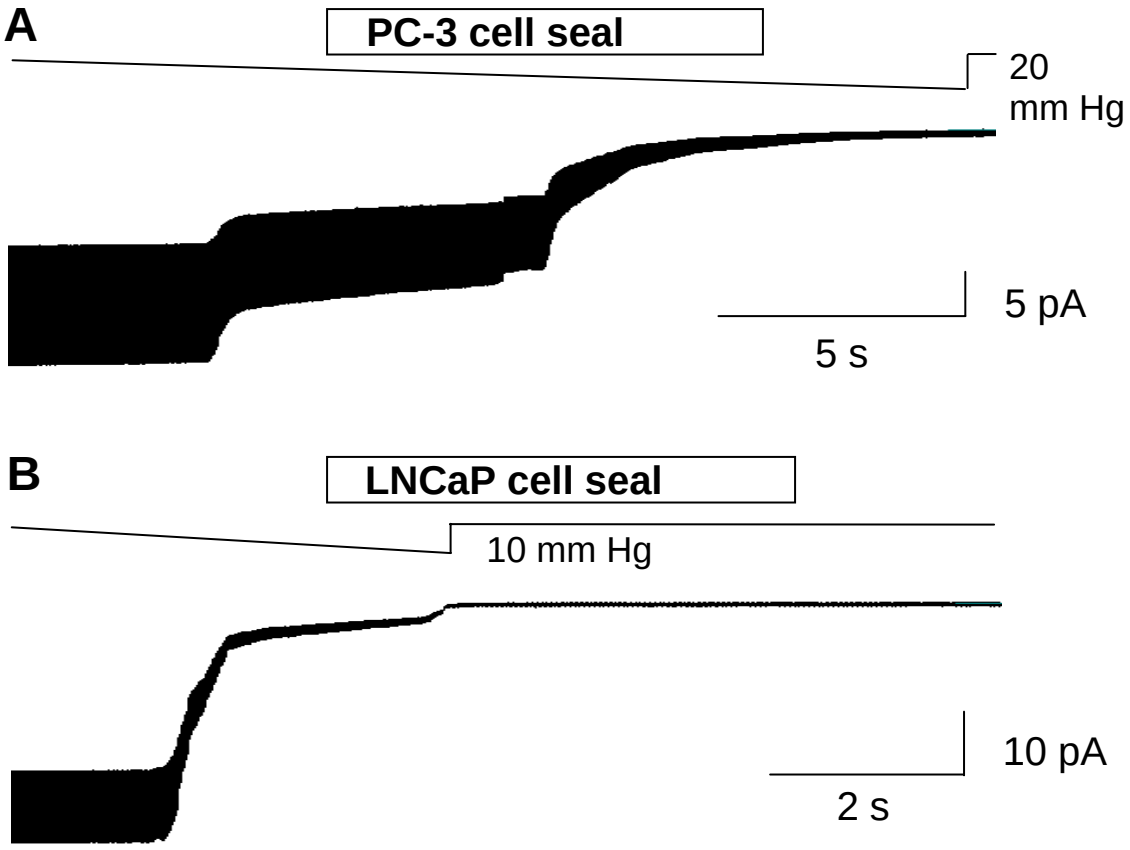

Figure 9. PC3 cells require longer and stronger suctions in order to form tight seals compared with LNCaP seals, which presumably reflects a less compliant membrane-CSK complex in PC-3 cells.

Task 1.4 is completed. We have excluded a dominant role for actin and membrane cholesterol in regulating MscCa gating. Results of our experiments indicate that the effects of different intrinsic membrane compliance of PC-3 and LNCaP cells on sealing is the strongest determinant of gating as measured by cell-attached patch recording.

Task 2: Determine the effects of cancer-related conditions that promote tumor progression to increased invasiveness on MscCa properties.

2.1 Use Westerns, immunofluorescence and patch clamp recording to examine the influence of TNF-α, a transcriptional regulator of TRPC1 expression, and TGF-β both known to promote the EMT on MscCa properties in NPE and PC cells.

Time line: Year 2, months 1-9.

Milestone: TNF-α and TGF-β regulation of MscCa/TRPC1 expression in PT cells.

2.2 Use time-lapse Ca^{2+} imaging to determine the effects of transforming factors on the functional properties, motility and $[Ca^{2+}]_i$ dynamics of NPE and PC cells.

Time line: Year 2, months 6-12, Year 3, months 1-12.

Milestone: TNF-α and TGF-β effects on $[Ca^{2+}]_i$ dynamics and cell migration.

We have delayed the TNF-α and TGF-β experiments because they will have more meaning when we identify the protein forming MscCa given we have excluded a direct role of TRPCs.

2.3 Use various conditions know to trigger increased invasiveness in the normally noninvasive LNCaP cell line to study the role of MscCa in triggering progression in these sub-lines.

Time line: Year 1, months 1-12; year 2 months 1-9.

Milestone: Identify cancer-linked progression factors that act on MscCa.

We have tested 3 different LNCaP sub-lines that have been reported in the literature to show increased invasiveness over the parent LNCaP cell. One sub-line was generated in our laboratory by long term growth in the absence of androgen using charcoal-depleted serum over several months (Tso et al., 2000). Under these conditions many of the LNCaP did not survive and those that did developed long processes. However, they did not show significant locomotion and patch clamp studies indicated MscCa currents with similar peak amplitudes and kinetics as untreated LNCaP cells. We have also tested a LNCaP cell sub-line generated by in vivo selection (Wu et al., 1998) and by transfection with β3 integrin subunit (Zheng et al., 1999). However, again neither sub-line showed significant migration as judged by time-

lapse video microscopy and the MscCa activity was indistinguishable for the parent LNCaP sub-line.

Deliverables: Regulator of MscCa expression in PT cells.

Our results indicate that none of published treatments of LNCaP cells, including long term culture in the absence of androgen, *in vivo* selected LNCaP subline, or β3 integrin that have been reported to increase in vivo invasiveness of transplanted LNCaP cells alter MscCa conductance or kinetics. Nor do they stimulate LNCaP cells to switch to a migratory mode.

Task 3: Quantify the ability of treatments that selectively target MscCa/TRPC1 and that block PC-3 cell migration in vitro to block PC-3 cell invasion when orthotopically implanted in nude mice.

3.1 Use time-lapse videomicroscopy and patch-clamp techniques to characterize the motility of eGFP-transfected PC-3 cells in which MScCa/TRPC1 has been selectively over-expressed or silenced.

Time line: Year 1, months 1-12.

Milestone: Genetic block of PC-3 cells migration.

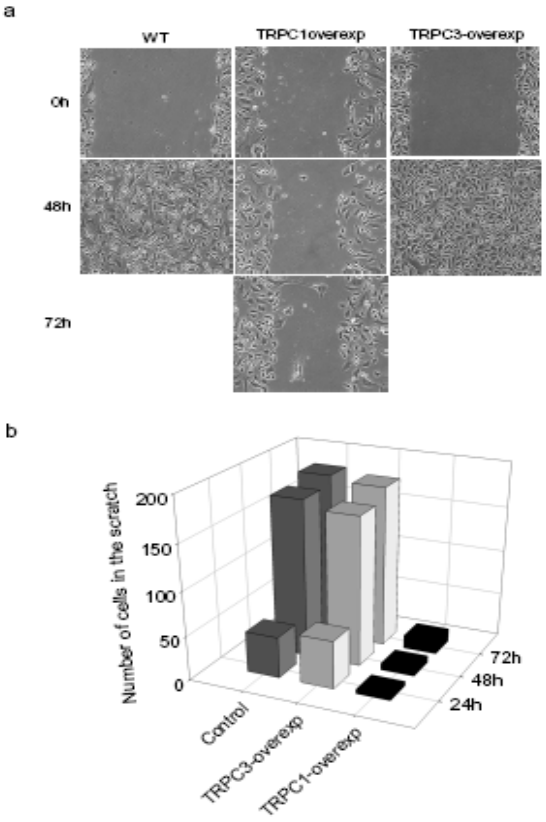

Figure 10. Wound/scratch closure assays of control PC-3 cells and PC-3 sublines that permanently over expresss TRPC1 or TRPC3. Overexpression of TRPC1 but not TRPC3 blocks PC-3 cells migration.

The results shown in Figures 6 and 10 indicate the successful generation of PC-3 cell sub-lines in which specific TRPC expression is altered. We have completed the characterization of these sub-lines in terms of MscCa expression and motility.

3.2 Carry out orthotopic implantation of eGFP-labeled human PC-3 cells in which MscCa expression and test the effects on tumor invasion and metastasis as measured using fluorescence.

Time line: Year 2, months 6-12, year 3, months 1-12.

Milestone: Block of the PC and invasion and metastasis disease *in vivo*.

Deliverables: New gene constructs that can block PC invasion and metastasis.

This task has been compromised by our new findings that TRPC do not form MscCa. Before this insight, in year 2 we implanted siTRPC1-PC-3 cells subcutaneously in nude mice as a first step in carrying out orthotopic implants in mice. As indicated in Figure 11 the PC-cell tumors grew but their fluorescence was less than the autofluorescence. The weak fluorescence of our GFP transfected PC-3 cells contrasts with the strong fluorescence seen with the eGFP-PC3 cells that were virally transfected with the cDNA for GFP by Anticancer Inc. (Figure 12).

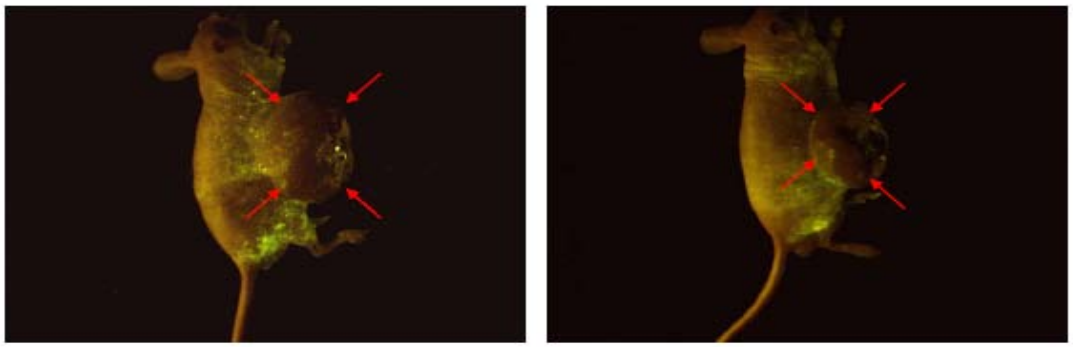

Figure 11. Two mice that were subcutaneously injected with the eGFP-shTRPC1-PC3 cell subline. The red arrows delineate the tumor that does not show significant fluorescence above background fluorescence.

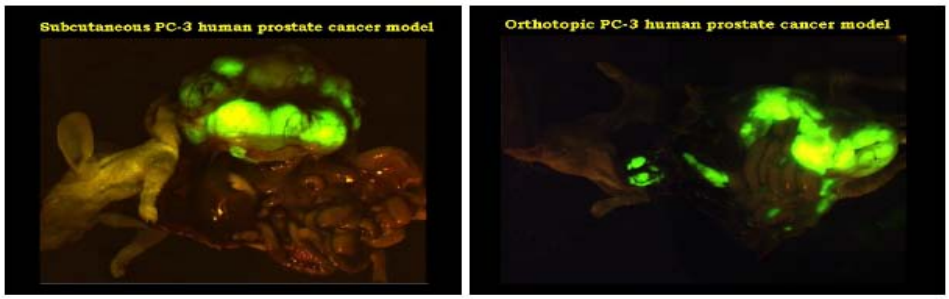

Figure 12. Shows the bright fluorescence of PC-3 cells virally-transfected with cDNA of GFP and injected subcutaneously (left) or implanted orthotopically (right) by Anticancer inc.

To overcome this limitation we initially transfected the short hairpin TRPC1 construct into a virally transfected GFP PC-3 cell sub-line, and have selected a sub-clone using antibiotic resistance. The new eGFP-siTRPC1 PC3 sub-line did show stronger fluorescence compared with the previous sub-line (Fig. 13). Anticipating that the wild type PC3 cell would not be a proper control for off target effects of siRNA transfection we also generated a cell control cell line that had been transfected with scrambled siRNA. However, this control cell line also showed a cell motility that was reduced compared with the wild type PC-3 cell.

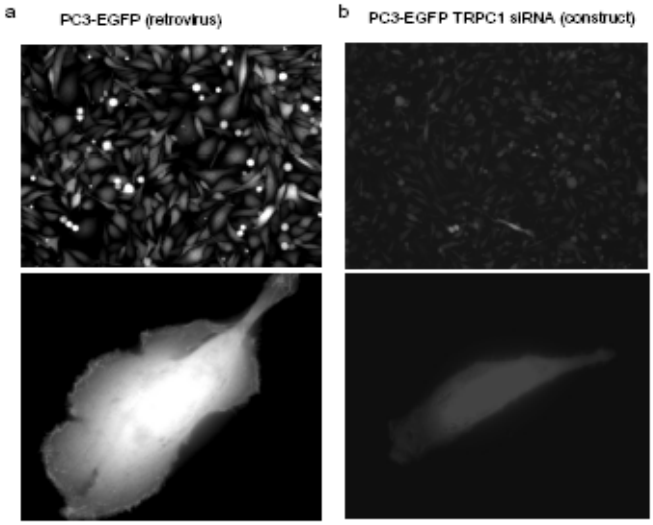

Figure 13. Comparison of the fluorescence of a siTRPC1 PC-3 cell (left panels) that was permanently transfected by retrovirus to express GFP and our earlier PC-3 cell line transfected with GFP-siTRPC1 using lipofection.

Task 3.2 has been attempted as originally intended. Anti-cancer Inc is willing to carry out another *in vivo* tumor growth assay and a orthoptopic impanation of our treated and control PC-3 cells to test the role of MscCa. However, we intend to utilize this assay when we resolve the protein identity of MscCa rather than pursue the assay for TRPC1 suppression.

KEY RESEARCH ACCOMPLISHMENTS

Our key research accomplishments in years 1 to 4 are as follows: (the new results from Year 4 are bolded).

1. MscCa is expressed in human prostate tumor cells.

2. MscCa differs in its density and surface distribution between invasive and noninvasive prostate tumor cells.

3. MscCa as measured by patch recording is expressed uniformly on the surface of PC-3 on both the front lamellipodia and the rear tether of migrating prostate tumor cells.

4. In contrast to the uniform distribution of MscCa, TRPC1 is expressed in a punctuate pattern that is concentrated on the trailing tether of PC-3 cells.

5. MscCa is expressed at a higher patch density in LNCaP cell lines and its sublines.

6. Internal Ca2+ stores located in the endoplasmic reticular are more concentrated in the cell body than in the front lamellipodia of the migratory PC-3 cell but uniformly expressed in the non invasive LNCaP cell.

7. The polarized distribution distribution of internal Ca2+ stores can account for the intracellular Ca2+ gradient (high rear-low front) that develops in migrating prostate tumor cells and determines migration directionality.

8. Suppression or over expression of TRPC1 and suppression of TRPC3 blocks prostate tumor cell migration as measured by time-lapse video microscopy of wound closure assay.

9. Suppression of TRPC1 and TRPC3 and overexpression of TRPC1, 3 and 6 does not alter MscCa currents when compared with transfected controls.

10. The reported role of TRPM7 in forming MscCa in PC cells is not supported by our immunological and knock down experiments.

11. Both the magnitude and temporal characteristics of mechanical stimuli applied to the tumor cells can affect the transition between the non motile and motile MscCa gating mode.

12. The different sealing protocols required to make tight seals on PC-3 cells compared with LNCaP cells (i.e. stronger and more prolonged suction for PC-3 cells) due to their different membrane compliance may be a significant determinant of the gating mode recorded with cell-attached patch recording.

13. Our original hypothesis that changes in the actin cytoskeleton is critical in mediating the transition in MscCa gating from the noninvasive to the invasive mode is not supported by experiments testing various agents known to promote either actin deploymerizaion or actin polymerization.

14. Changes in membrane fluidity induced by cholesterol depletion does not alter MscCa gating mode.

15. Different LNCaP cells sublines that have been reported to show increased invasiveness failed to show migratory behavior when measured in vitro by either wound closure assay or time lapse video microscopy. At least consistent with our hypothesis, these LNCaP cell sublines displayed the same MscCa properties haracteristic of the parent cell line.

16. In addition to blocking prostate tumor cell migration, TRPC1 suppression also inhibits prostate tumor proliferation thereby blocking tumor growth when implanted subcutaneously in nude mice.

17. Overexpressed YFP-TRPC1 is localized at the leading edge of PC-3 cells but is also concentrated around the cell nucleus. This result differs from the endogenous TRPC1 expression that is located on the trailing tether

18. Overexpression of TRPC1as verified by three different transfection vectors and by three independent motility assays reduces PC-3 cell motility.

19. Specific TRPCs regulate PC cell migration. However, by mechanisms other than altering MscCa expression.

REPORTABLE OUTCOMES

1. Maroto, R.; Hamill. MscCa regulation of tumor cell migration and metastastis, Current Topics in Membrane Transport, 59; 485-509, 2007.

2. Hamill, O.P.; Maroto, TRPCs as MS channels. Current Topics in Membrane Transport, 59; 191-231, 2007.

3. Kurosky, A. Maroto, R. Hamill, O.P. The stretch-activated calcium channel as a central regulator of prostate cancer cell migration. IMPaCT meeting; Innovative Minds in Prostate Cancer Today**;** Atlanta, Georgia; September 5-8, 2007

4. Gottlieb, P,, Folgering, J,, Maroto, R,; Raso, A,; Wood, T.G,; Kurosky, A,; Bowman, C,; Bichet, D, Patel, A, Sachs, F, Martinac, B, Hamill, O.P., Honore, E. Revisiting TRPC1 and TRPC6 mechanosensitivity. Pflugers Archive, 455; 1097-1103, 2008.

5. Hamill, O.P. Maroto, R. Mechanically-ignited Ca^{2+} flickers: Reemergence of an old role in mechanosensitiveregulation of cell migration and the discovery of a new role in chemotaxis. Cell Science Reviews. 6 (2), 2009.

6. Maroto, R. Kurosky, A., Hamill, O.P. Controversies related to stretch-activated mechanosensitive Ca2+ permeable channels. Abstract of talk presented at HHMI sponsored meeting and Janelia Farm Campus. “Force-gated ion channels” Page 31, May 18-21, 2008.

7. Maroto, R. Kurosky, A., Hamill, O.P. The role of stretch-activated and TRPC channels in prostate tumor cell migration. Invited speaker abstract published on the web prior to Keystone symposium. “The role of Mechanotransduction in physiology and disease”. Jan 18-24, 2009.

8. Hamill, O.P. Maroto, R. Stretch-activated calcium channel is required for prostate tumor cell migration. IMPACT Conference “Innovative Minds in Prostate Cancer Research Today” Orlando March 9-12 (2011).

9. Maroto, R. Kurosky, A. Hamill, O.P. Expression and function of canonical transient recptor potential channels in human prostate tumor cells. IMPACT Conference Innovative Minds in Prostate Cancer Research Today presentation Orlando March 9-12 (2011).

CONCLUSIONS

The completion of the experiments of years 1 and 4 confirms that MscCa is a promising new target to block prostate cancer progression and provides added motivation to further understand its detailed mechanisms of action in vitro and in vivo. In particular, because Ca2+ influx via MscCa appears important in regulating all major modes of cell migration

(i.e., mesenchymal, amoeboid and collective) it may be more effective than other strategies targeting integrins and metalloproteinases that appear to fail in vivo as a consequence of migration mode plasticity (Wolf & Friedl, 2006, Maroto & Hamill, 2007). The protein nature of MscCa in PC cells remains unresolved but results of experiments in this proposal now exclude a role for specific TRP channels even though our other experiments indicate that TRPC1 and TRPC3 expression are required for PC cell migration

CONTRACTUAL ISSUES: Information provided in this final report supports the following:

Task 1.1	Months 1-6	Completed
Task 1.2	Months 1-12	Completed
Task 1.3	Months 1-6	Completed
Task 1.4	Months 18-24	Completed
Task 2.1	Months 13-20	Incomplete
Task 2.2	Months 18-36	Completetd
Task 2.3	Months 1-18	Completed
Task 3.1	Months 1-12	Completed
Task 3.2	Months 18-36	Completed

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Copies of the manuscripts (1 under revision) and meeting abstracts (including two for the Orlando IMPACT meeting in March 2011) published during the DOD funding period Dec. 2008 – March 2011 are listed in Appendix .

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Dr. Rosario Maroto
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INVITED REVIEW

Twenty odd years of stretch-sensitive channels

O. P. Hamill

Received: 14 June 2006 / Accepted: 27 June 2006

Abstract After formation of the giga-seal, the membrane patch can be stimulated by hydrostatic or osmotic pressure gradients applied across the patch. This feature led to the discovery of stretch-sensitive or mechanosensitive (MS) channels, which are now known to be ubiquitously expressed in cells representative of all the living kingdoms. In addition to mechanosensation, MS channels have been implicated in many basic cell functions, including regulation of cell volume, shape, and motility. The successful cloning, overexpression, and crystallization of bacterial MS channel proteins combined with patch clamp and modeling studies have provided atomic insight into the working of these nanomachines. In particular, studies of MS channels have revealed new understanding of how the lipid bilayer modulates membrane protein function. Three major membrane protein families, transient receptor potential, 2 pore domain K^+, and the epithelial Na^+ channels, have been shown to form MS channels in animal cells, and their polymodal activation embrace fields far beyond mechanosensitivity. The discovery of new drugs highly selective for MS channels ("mechanopharmaceutics") and the demonstration of MS channel involvement in several major human diseases ("mechanochannelopathies") provide added motivation for devising new techniques and approaches for studying MS channels.

Keywords Mechanosensitive channels · Mechanotransduction · Transient receptor potential · Patch clamp · Giga seal

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Introduction

The formation of the giga-seal depends upon applying suction to draw the membrane into the pipette. Once the seal is formed, suction is not necessary, and it should be released [53]. However, the membrane patch spanning the pipette can now be mechanically stimulated by hydrostatic or osmotic pressure gradients applied across the patch without disrupting the seal. This feature allowed the first recordings of cell-swelling and stretch-activated channel currents [50, 52]. Over the last 20-odd years, interest in mechanosensitive (MS) channels has progressed from being a possible patch clamp recording artifact to a central player in our understanding of protein–bilayer interactions and a promising new therapeutic target against several major human diseases. This article highlights some recent developments and unresolved issues regarding MS channels, with a major focus on the MS Ca^{2+} permeant cation channel (MscCa) recently identified in vertebrate cells [97].

What happens to the membrane patch in the pipette?

An important issue for MS channels is how the process of aspiration and sealing of the membrane in the pipette alters the mechanics and possible stretch sensitivity of channels in the patch. Because of the small size and inaccessibility of the patch in the pipette, a variety of techniques, including high-resolution video imaging [121, 152–154, 184], high-voltage electron microscopy [142], atomic force microscopy [70], and fluorescence-imaged microdeformation [32, 33] have been used to study the aspirated patch and its underlying cell cytoskeleton (CSK). Here we focus on results obtained on the *Xenopus* oocyte [182–185]. The first issue is whether the pressure/suction applied to the patch after seal formation somehow

induces changes in the seal resistance that appear as "MS-channel-like" events. This idea is not entirely far-fetched because video imaging indicates that suction tends to peal the membrane off the walls of the pipette [121], and gated, cation-selective channels have been recorded with patch pipettes sealed onto noncellular hydrophobic surfaces [145]. However, although strong suction can rupture the patch, it typically does not disrupt the giga-seal, thereby allowing for tight seal whole-cell and/or outside-out patch recording [53]. In addition, suction ramps applied to cell-attached frog oocyte patches reversibly activate either a saturating macroscopic current (Fig. 1a) or a unitary amplitude current event (Fig. 1b) depending upon patch area [60]. These current waveforms are consistent with multiple and single MS channel patches, respectively, but difficult to reconcile with MS changes in seal resistance. Even more compelling is that patches formed on pure liposomes fail to express MS channel currents [97, 102, 122, 160]. This absence allowed for the identification of MS channel proteins following functional reconstitution of solubilized membrane proteins from bacteria and archaea [81, 158, 159] and, most recently, from *Xenopus* oocytes [97].

Although MS channels are clearly not seal "leaks," the sealing process does change patch geometry and the underlying CSK, thereby altering patch mechanics. For example, Fig. 2a and b shows electron microscopy (EM) images of the *Xenopus* oocyte surface, indicating its extensive membrane folding and high density of microvilli (est. ~7 microvilli per square micrometer) [181, 184, 185]. This complex membrane geometry is also reflected in electrical capacitance (C_m) measurements that indicate a membrane surface area that is tenfold greater than that required for the cell's volume [184]. In contrast, high-resolution video images of cell-attached oocyte patches (Fig. 2c, d) indicate an optically smooth membrane that is pulled flat and perpendicular to the walls of the pipette [184; see also 113, 152, 153, 161]. Furthermore, C_m measurements indicate a patch area of approximately 50 μm^2, consistent with the patch geometry but inconsistent with the approximately 500 μm^2 expected if the approximately 300 microvilli and membrane folds evident in Fig. 2a and b were preserved during the sealing process [184]. Presumably, the suction used to obtain the giga-seal is sufficient to smooth out the surface folds and microvilli so that the cell membrane is now tightly stretched over an expanded CSK (Fig. 3). This smoothing out of microvilli is not an exclusive patch clamp phenomenon because a similar phenomenon has been visualized in EM studies of cells undergoing osmotic inflation [82] and spreading before cell migration [37]. In these cases, the process is presumably reversible, indicating the considerable plasticity of the microvilli and their supporting CSK.

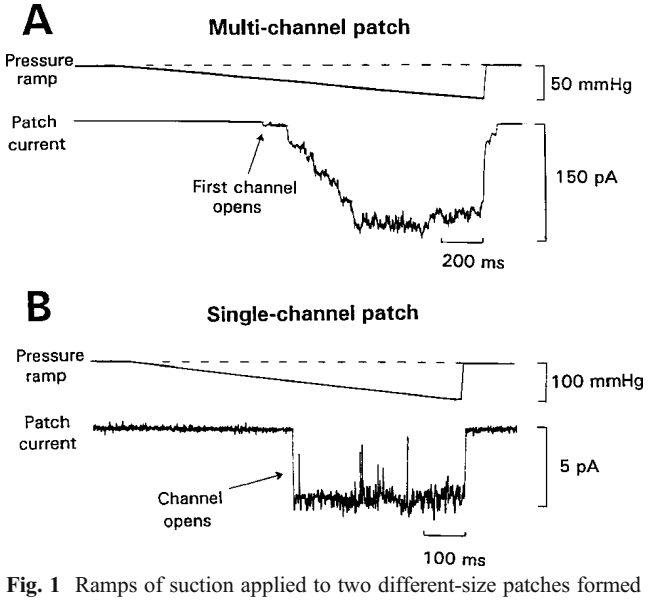

Fig. 1 Ramps of suction applied to two different-size patches formed on a *Xenopus* oocyte are consistent with a finite number of discrete MS channels but inconsistent with pressure-induced leaks in the seal. **a** A relatively large patch formed with an approximately 3-μm-diameter tip pipette shows a current that fully saturates at around 40 mmHg. The *arrow* indicates the initial opening of 5 pA single MscCa. **b** A smaller patch formed with an approximately 0.50-μm-diameter tip pipette reveals the opening of single MscCa, indicating that once open, the channel current was independent of suction. These results indicate that MscCa is either open or closed, and the saturation of current in a multichannel patch represents the balance between opening and closing rates for a finite number of channels

There are at least two related mechanisms by which changes in patch geometry will increase stretch sensitivity of channels in the patch. First, in the absence of any excess membrane, brief pressure pulses applied to the patch will rapidly flex the membrane and increase bilayer tension (Fig. 2c, d). The flexing of the membrane either outward with suction or inward with pressure results in the rapid activation (<1 ms) of inward channel currents [107, 108, 184]. In contrast, more sustained pressures would be required to inflate the oocyte and smooth out the membrane reserves to increase membrane tension (T_m) [15, 117, 184]. Second, according to Laplace's law, $P=2\ T_m/r$, the pressure (P) required to activate channels in the patch with a radius of curvature (r) of approximately 1 μm would be 1/20th of that required to activate the same channels located on microvilli that have a radii of curvature of approximately 0.05 μm. For example, stimulus–peak current relations shown in Fig. 4 indicate that half the channels in an oocyte patch are activated by a suction of approximately 10 mmHg (~1.3 kN m^{-2}), which translates to a tension of approximately 0.6 mN m^{-1} (i.e., the near-symmetrical suction/pressure relations indicate a tension-gated channel and justify Laplace's law). To achieve the same tension in microvilli would require a suction of 200 mmHg. However, this would exceed the approximately 100-mmHg that causes patch rupture (i.e., a lytic tension of ~6 mN m^{-1}) under these conditions [119].

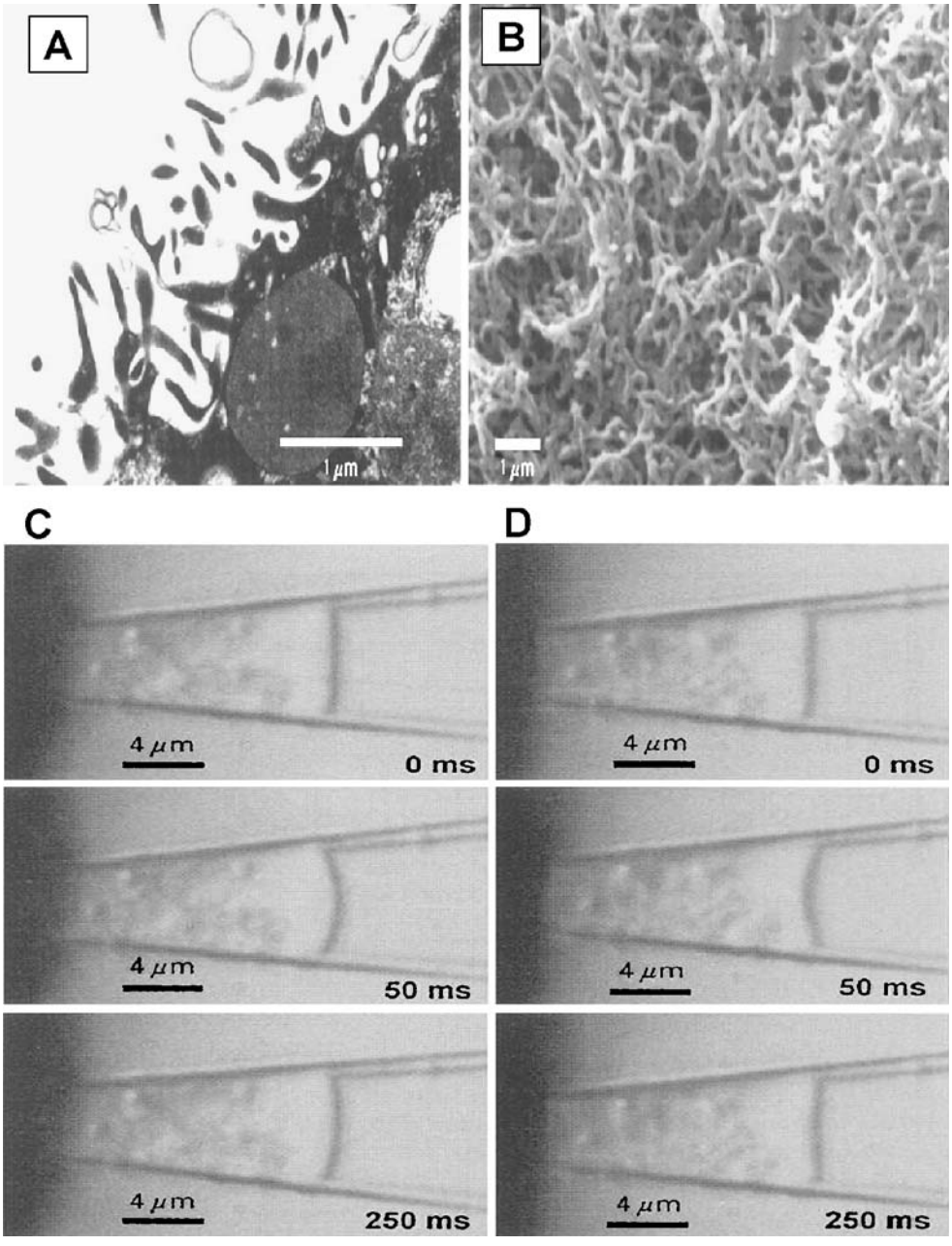

Fig. 2 Comparison of the membrane geometry of the oocyte surface and of the patch sealed in a pipette. **a** Transmission EM of the oocyte surface showing prominent microvilli containing dark cytoplasmic material. **b** Scanning EM of the oocyte surface, indicating the high density of microvilli. **c** High-resolution video images of a membrane patch before (0 ms), during (50 ms), and after (250 ms) a 100-ms suction step. **d** The same patch as in C except a 100-ms pressure step was applied. Both suction and pressure steps activated a 50-pA inward current (modified from Zhang and Hamill [184] and Zhang et al. [185] with permission)

The sealing process may also alter patch mechanics by changing the CSK structure underlying the patch [55, 63, 173]. When a gentle sealing protocol is used to achieve the giga-seal, the cortical CSK network that is pulled into the pipette may be dilated without disrupting its links with the membrane [54, 185; see also 32, 33]. However, if the suction used to draw the membrane into the pipette exceeds the strength of CSK–membrane linkages, then a CSK-free membrane (bleb) may be formed [55, 63, 112]. Similarly, after the membrane has sealed in the pipette, additional suction may cause the blebbing at the membrane cap as shown in Fig. 5 [185]. This membrane blebbing can either increase or decrease the stretch sensitivity depending upon the specific MS channel. For example, the TREK and TRAAK MscK channels show an increase in stretch sensitivity, presumably because the CSK normally acts as a constraint and prevents tension being conveyed to the bilayer [69, 151]. On the other hand, MscCa typically shows a loss of both stretch sensitivity and fast dynamics presumably because they depend upon CSK interactions with the channel/membrane (Fig. 5) [55, 156, 185].

In summary, giga-seal formation introduces significant changes in patch mechanics that can alter the mechanosensitivity of channels in the patch. The extrinsic changes in membrane geometry and CSK structure may have different effects on specific channels depending upon

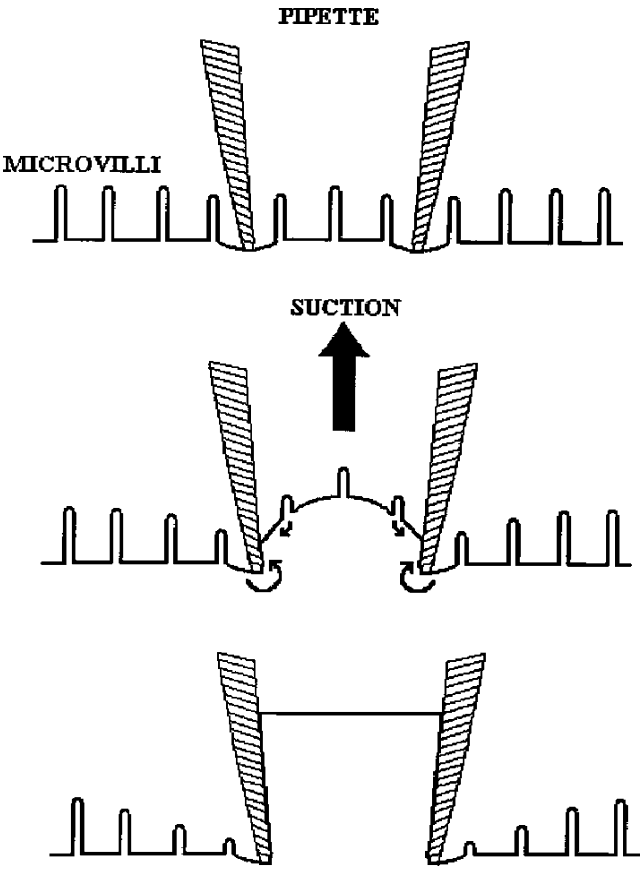

Fig. 3 Schematic illustrating the proposed smoothing out of microvilli caused by the pipette aspiration and giga-seal formation

their intrinsic properties (i.e., protein structure and protein–CSK interactions).

Do animal cells generate/experience membrane tensions that activate MS channels?

Although it has been questioned whether animal cells experience the same in-plane membrane tensions that activate MS channels in the patch [30, 149, 172], there is evidence that they can experience even larger tensions that result in membrane rupture. In particular, cell wounding events, as judged by the cell filling with the membrane impermeant fluorescein-labeled dextran, have been observed in experimentally unperturbed rodent skin, gut epithelium, cardiac, and skeletal muscle, and found to increase in frequency with mechanical loading [11, 109–111, 164]. The cell types that commonly experience wounding in vivo include epidermal cells and fibroblasts (skin), epithelial cells and smooth muscle (GI tract and respiratory system) endothelial cells, and smooth muscle (cardiovascular system) and peripheral neurons. The proportion of the cells wounded in the various systems can range from 2 to as high as 25%. In eccentrically exercised muscle (e.g., downhill running), there can be a tenfold increase in cell wounding compared with resting muscle, and in dystrophic mice that lack the CSK–structural protein dystrophin, exercise can produce massive wounding events that ultimately overload the muscle regeneration mechanisms [180]. Normal migrating cells can generate traction forces that not only lengthen and stretch the cell but also cause cell fragments to be ripped off and deposited along the migration trail [104]. Furthermore, if the normal contractile mechanisms that allow a migrating cell to retract its rear are blocked, the front of the cell can tear away from the cell body and move off as a motile cell fragment [170]. The common occurrence of these traumatic mechanical stresses under physiological and pathological conditions has presumably provided strong selective pressure for the evolution of the membrane resealing mechanism(s) that is widely expressed in eukaryotic cells [11, 109–111, 164].

Despite the above evidence of membrane rupture tensions (i.e., >5 mN m^{-1}), direct estimates of membrane tension in "resting" isolated cells indicate much lower values (<0.1 mN m^{-1}) [28–31, 116, 138]. The tension measurement involves pulling a tether from the cell surface using optical tweezers and measuring the force required to maintain it at constant length. The basic assumption is that membrane tension is contiguous over the whole surface so that pulling a tether from one region perturbs the tension in all regions of the cell membrane. From the static tether force (F_0) measured in piconewtons, one can estimate the membrane tension T_m according to the equation:

$$F_0 = 2\pi(2BT_m)^{1/2}$$

where the membrane bending stiffness (B) is assumed to be constant with a value of 2.7×10^{-19} N m^{-1}. The practical limitation of this technique is that the optical tweezers can only sustain forces up to 100 pN, which would correspond to a tension of 0.5 mN m^{-1}. For an animal cell with its cortical CSK, the measured tension is assumed to represent a combination of in-plane tension and CSK adhesion, and is referred to as the "apparent" tension. However, T_m measurements of membrane blebs that lack CSK indicate the in-plane tension contributes only 25% of the T_m value [28]. Experiments on two different cell types (RBL 2H3 cells and snail neurons) indicate that cell swelling increases steady-state tensions from approximately 0.04 to 0.12 mN m^{-1},which then returns to approximately 0.04 mN m^{-1} with reshrinking [30, 31]. However, the same cells also experience tension surges that exceed the strength of the trap (i.e., >0.5 mN m^{-1}). Based on other experiments measuring exocytosis/endocytosis as a function of membrane tension, it has been proposed that membrane surface area and tension are in a feedback loop in which high tensions favor membrane recruitment, and low tension favors membrane retrieval [31, 116, 148]. As a conse-

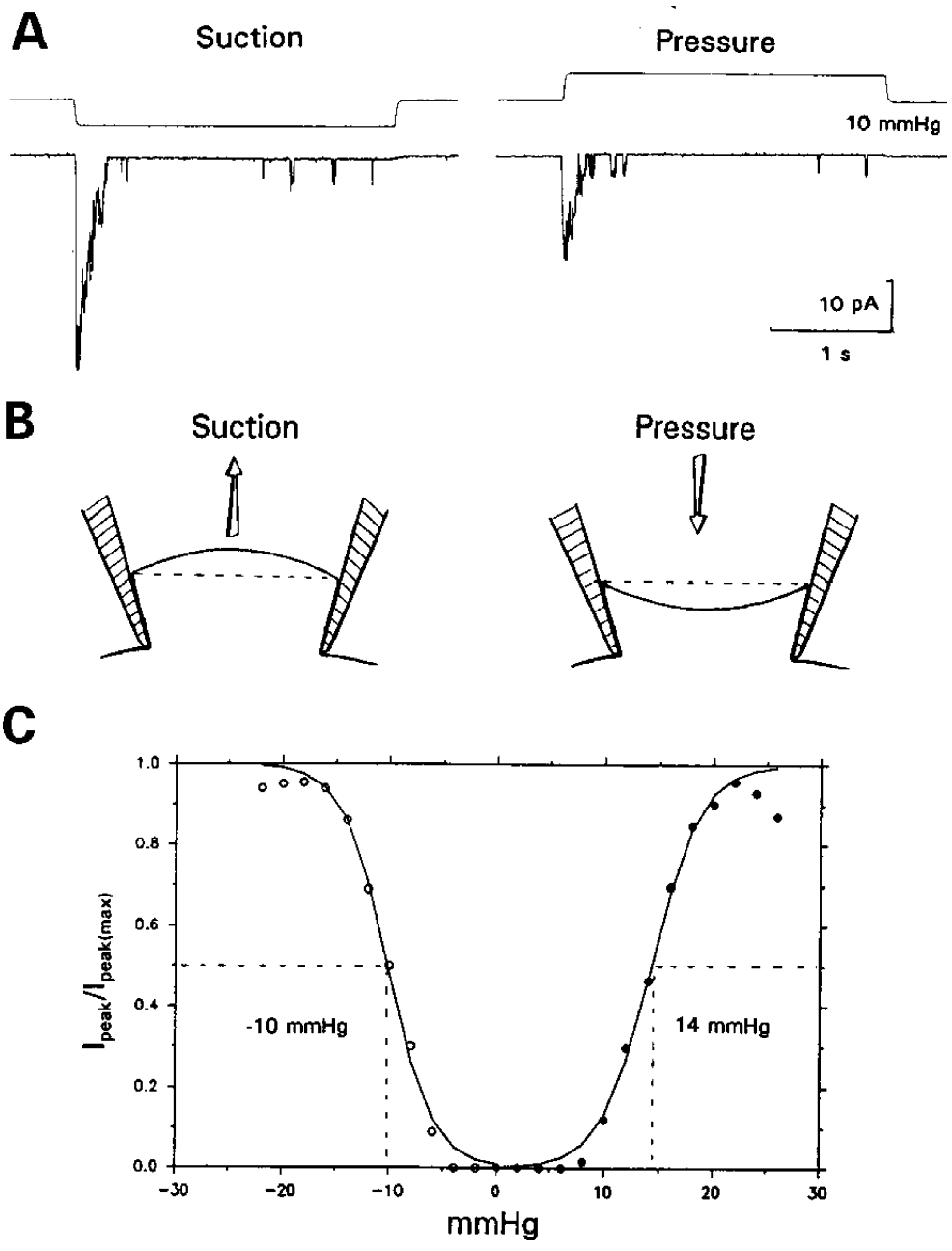

Fig. 4 Inward current responses to suction and pressure pulses applied to an oocyte patch. **a** Both suction and pressure pulses (2.5 s) result in rapid opening of MscCa that mostly close within 200 ms of the pulse. **b** Schematic showing flexion of the patch outward (suction) or inward (pressure). **c** Stimulus–response relations for suction and pressure steps. The sigmoid fits indicate that suction ($P_{0.5}$=−10 mmHg) was slightly more effective than pressure ($P_{0.5}$=14 mmHg) in activating MscCa

quence, it has been proposed that surface area regulation (SAR) maintains membrane tension around a relatively low set point of approximately 0.1 mN m^{-1}, which would be well below the lytic tension (≥5 mN m^{-1}) and the near-lytic tensions (~4 mN m^{-1}) required to activate the bacterial MS channels. However, the direct measurement of high tension surges exceeding the low set point and the occurrence of cell wounding events indicate that SAR mechanisms can be saturated. Furthermore, as indicated in Fig. 6, lower tensions are required to activate MS channels in animals cells ($T_{50\%}$ for MscK=2.4 mN m^{-1}) and (MscCa ~0.6 mN m^{-1}) compared with MscL (~4.7 mN m^{-1}) that functions as a last-resort safety valve [94]. As a consequence, MS channels in animal cells would seem more geared to regulating processes with lower tension set points such as regulatory volume decrease [20, 26, 146, 147, 169] cell locomotion [92] and, possibly, SAR via Ca^{2+}-induced exocytosis. However, it appears that integrins rather than MscCa act as the mechanosensor for MS exocytosis/membrane trafficking at the frog neuromuscular junction [24] and the oocyte [96].

"Mechanopharmaceutics"

Progress in the MS channel field would be greatly enhanced by the discovery of high-affinity agents that

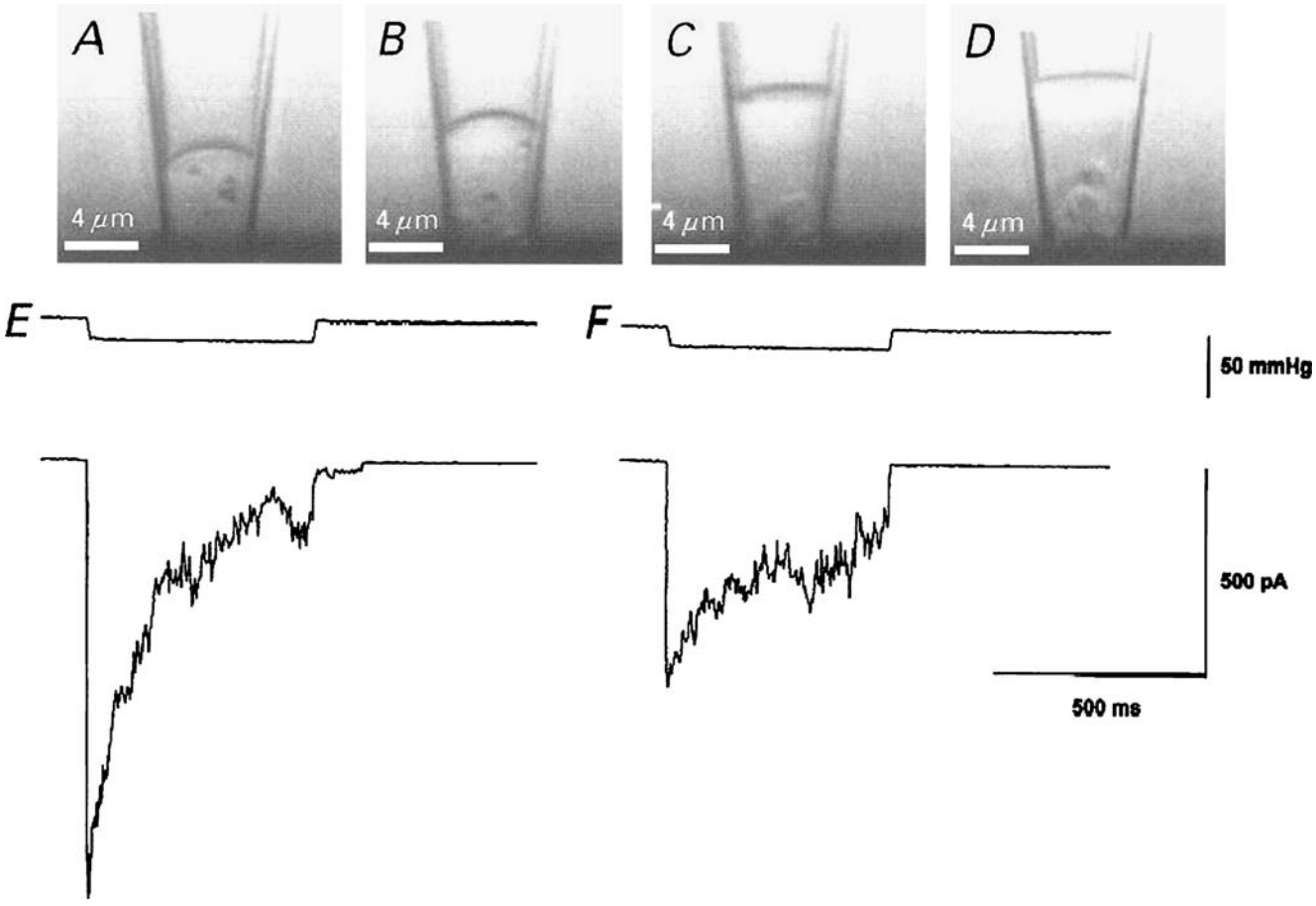

Fig. 5 Changes in the membrane and patch currents as a consequence of repetitive pressure pulses applied to the patch pipette. **a**–**d** video images of a cell-attached patch at different times after formation of the giga-seal. Between each image, suction steps (20 mmHg, 500 ms) were applied. **a** The first image taken immediately after giga-seal formation shows the patch curved outwards and located closed to the end of the pipette (~5 μm). Particles located in the cytoplasm exhibited no motion presumably because they were still constrained by the intact CSK. **b–d** repetitive suction pulse caused the patch to move up the pipette away from the cell, and a clear space developed between the membrane and the CSK remaining close to the cell. Particles that moved into this space displayed Brownian motion, indicating the loss of constraining CSK structures [153]. **e** Application of suction pulses at **a** caused a rapid opening of MscCa that closed almost completely. **f** Application of a suction pulse at **d** caused activation of a smaller more sustained current (from Zhang et al. [185] with permission)

selectivity target specific MS channels. These agents would be highly useful for following MS channel proteins during purification procedures and identifying MS channel roles in novel functions. In addition, given that MS channels may be polymodally activated (e.g., by tension, voltage, pH, temperature, Ca^{2+} store depletion, and/or lipid second messenger) [27, 125, 171], it would be advantageous to discover agents that selectively acted on MS channel only when they were mechano-gated. Although no ideal MS channel reagent has yet been discovered, a number of compounds have been identified that act as MS channel blockers or activators [48, 61, 65]. One class, amiloride and its analogs, appear to act on a traditional "lock and key" protein receptor, whereas other agents, GsMTx-4 and possibly maitotoxin, seem to act via nontraditional "receptors" at the lipid or lipid–protein interface where they may change the local bilayer mechanics and thereby modify MS channel gating. Below we briefly review their salient features.

Amiloride has been the most rigorously studied in terms of its MS channel blocking mechanism and provides an example where variations in mechanistic detail may enable discrimination between different channel families in terms of their participation in specific MS functions. In particular, the amiloride block of MscCa/TRPC-1 in *Xenopus* oocytes [56, 89–91] and MscCa in vertebrate hair cells [141] has been shown to involve basically the same unusual mechanism in which two amiloride molecules bind cooperatively to channel sites that only become accessible at hyperpolarized potentials after the channel has opened. This mechanism, referred to as "conformational" block, implies different open-state conformations at hyperpolarized vs

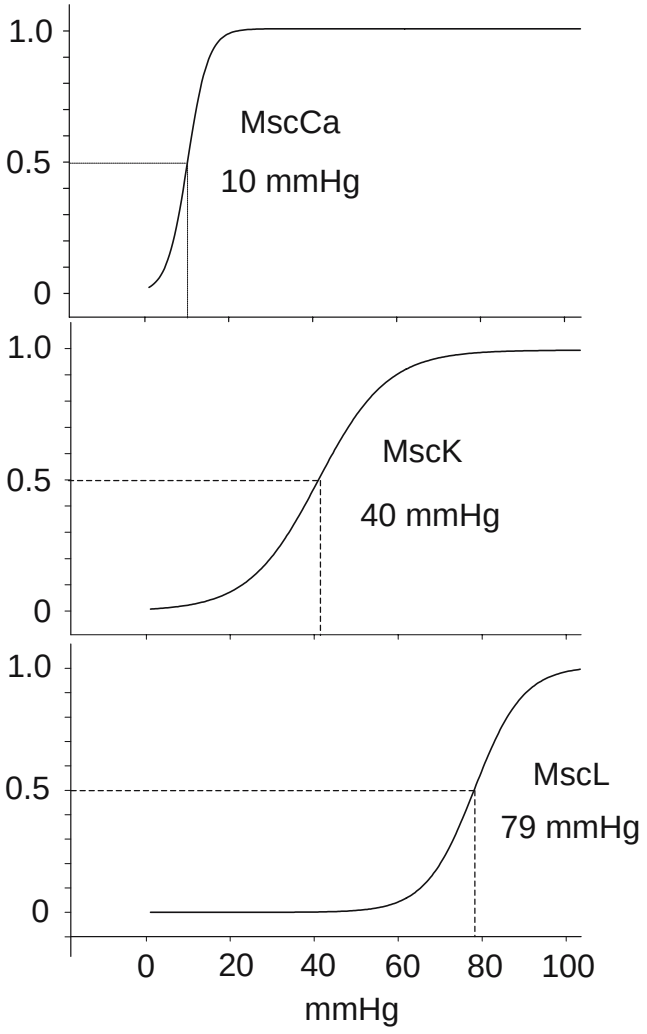

Fig. 6 Comparison of the normalized pressure–current relations for three different MS channels. MscCa curve fitted to curve Fig. 2c (*top*). MscK curve fitted to data from Ref. [69] (*middle*). MscL curve fitted to data from Ref. [54] (*bottom*)

depolarized potentials, and is distinctly different from the amiloride block of the high-affinity amiloride-sensitive epithelial Na^+ channel (ENaC) where the voltage dependency arises because the positively charged amiloride binds to a pore site that senses a fraction of the electric field [130]. A further difference is seen in the order of potencies of amiloride analogs in blocking the two channel classes (Table 1) [80]. Amiloride blocks the MEC-4 DEG/ENaC currents in touch receptor neurons in *Caenorhabditis elegans* [120] and also the mammalian arterial myogenic response, which has been used to implicate DEG/ENaC as the vascular mechanosensor [34]. However, amiloride also blocks TRPC-6, also implicated as the arterial smooth muscle mechanosensor [74, 175]. It will be interesting to determine if mechanistic differences in amiloride block (i.e., conformational vs pore block) can be used to resolve the MS Channel's identity.

Gadolinium blocks a variety of MS channels (e.g., MscCa, MscK, MscL, and MscS), several TRP channels (e.g., TRPC-1, TRPC-4, TRPC-5, and TRPV1), various voltage-gated (Ca^{2+}, Na^+, and K^+) and receptor-gated channels [e.g., *N*-methyl D-aspartate (NMDA), AChR, etc.] [61, 124, 136, 144, 165, 176, 178]. Because of its trivalency, Gd^{3+} will bind with high affinity to negative groups on proteins, lipids, and polysaccharides, as well as any inorganic anions present in solution [17, 61]. Its ionic radius (0.938 Å), which is similar to Na^+ (0.97 Å) and Ca^{2+} (0.99 Å), may also allow it to enter and bind to negatively change groups (Glu and/or Asp) within cation channels. Evidence that Gd^{3+} interacts directly with channel proteins comes from studies of specific TRP members (TRPV-1, TRPC-4, and TRPC-5) where Gd^{3+} has been shown to have dual effects, activating the channels at low micromolar concentrations (<100 μM) but blocking at higher concentrations (>300 μM). The activation of TRPV-1 depends upon binding to specific external glutamate residues that confer acid sensitivity on the channel, and neutralization of these residues blocks the activation and modifies inhibition [165]. Similar concentration-dependent potentiating and blocking effects also occur with TRPC-4 and TRPC-5 [77]. In contrast, Gd^{3+} only blocks TRPC-1 and TRPC-3 channels at relatively low micromolar concentrations [97, 166].

Table 1 Amiloride analog potency (IC_{50} amiloride/IC_{50} analog) of MS channels and the epithelial Na^+ channel

	Amiloride (IC50, μM)	DMA	Phenamil	PBDCB	Benzamil	HMA	I-NMBA	Reference no.
MscCa mouse hair cell	1 (53)	1.3	4.4	5	9.6	12.3	29	[141]
MscCa *Xenopus* oocyte	1 (500)	1.4	–	–	5.3	14.7 (BrHMA)		[90]
Epithelial Na^+ channel (high affinity)	1 (0.34)	0.04	17	–	9	0.04	7	[80]
Epithelial Na^+ channel (low affinity)	1 (10)	2.2	2.9	–	3.8	–	–	[80]

DMA Dimethylamiloride; *PBDCB* 5-(*N*-propyl-*N*-butyl)-dichlorobenzamile; *HMA* hexamethyleneamiloride; *I-NMBA* 6-iodide-2-methoxy-5-nitrobenzamile; *BrHMA* bromohexamethylene amiloride

An early view on how Gd^{3+} might block MS channels was via effects on the bilayer. Gd^{3+} has been shown to interact with black lipid membranes containing the negatively charged phosphatidylserine (PS) but not with the neutral phosphatidylcholine to increase the boundary potential and membrane tension [38]. However, whether these effects underlie MS channel block remains unclear because PS is normally restricted to the internal-facing monolayer, and Gd^{3+} acts externally. Gd^{3+} has also been shown to promote shape changes in giant unilamellar vesicles lacking PS [162]. In this case, it was proposed that Gd^{3+} bound to the hydrophilic lipid head (i.e., negative charge of the phosphate groups) of the external monolayer, and in doing so, decreased its surface area relative to the internal monolayer, thereby causing a change in membrane curvature. However, whether this effect would block MS channels remains unclear because amphipaths that also change membrane curvature usually result in MS channel activation [101, 135]. A recent study has reported that Gd^{3+} can block MS channels without altering pressure-induced changes in C_m, which would be expected if Gd^{3+} acted by altering membrane mechanics [156]. However, as pointed out by the authors, measurement of this parameter may be complicated because Gd^{3+} has multiple effects, including increasing the giga-seal [35].

GsMTx-4 is a 34-amino acid (4 kDa) peptide isolated from tarantula venom [48, 49, 155, 157]. It is amphipathic with a hydrophobic membrane face opposite a positively charged face, and it is a member of the inhibitory cysteine knot (ICK) toxin superfamily. GsMTx-4 is the most specific MS blocker identified to date. Because of its structure, it would be expected to be attracted to negative regions of proteins/lipids, and it will tend to partition into hydrophobic pockets either within the protein or at the protein/lipid interface. Unlike the nonspecific channel blocker Gd^{3+}, GsMTx-4 has so far not been reported to affect voltage- or receptor-gated channel. GsMTx-4 blocks MscCa at between 0.2 and 3 μM in chick heart, rat astrocyte, and human bladder and kidney cells [48, 49], and the crude tarantula venom also blocks MscCa in growing pollen protoplasts [36]. Most recently, GsMTx-4 has been shown to stimulate neurite outgrowth by blocking Ca^{2+} elevation in *Xenopus* spinal neurons [76]. However, GsMTx-4 does not block MscCa involved in auditory transduction [48], MscK formed by TREK (E. Honore, unpublished observations, cited in Ref. [48]) the bacterial MscL [99], and perhaps, most surprisingly, MscCa in *Xenopus* oocytes (R. Maroto and O.P. Hamill, unpublished observations). The last result is puzzling given that the oocyte MscCa is often treated as the prototypical MscCa, and TRPC-1 has been implicated as forming MscCa in the *Xenopus* oocyte and mammalian cells [97]. One possible explanation is that there are structural differences between MscCa proteins in different cell types. However, based on the observation that GsMTx-4 synthesized from D instead of L amino acids shows the same potency in blocking specific MS channels, it has been proposed that GsMTx-4 is more likely to act by binding to boundary lipids surrounding the channel, and, at least, consistent with this is that GsMTx-4 and its enantomer also alters the gating of gramicidin A, which is particularly sensitive to bilayer mechanics [157]. It may therefore be that differences in lipids between poikilotherms vs homeotherms is a factor that underlies the different GsMTx-4 sensitivities. In this case, it will be particularly interesting to determine GsMTx-4 action on MscCa/TRPCs reconstituted into defined lipid environments.

Maitotoxin (MTX) is a highly potent marine poison (LD 50 for mice 50 ng/kg) from the dinoflagellate (*Gambierdiscus toxicus)* that is responsible for Giguartera, a form of seafood poisoning [40]. It is water-soluble polyether with 2 sulfate esters, 28 hydroxyls, and 32 ether rings, and with a molecular weight of 3.4 kDa, it is the largest among the known nonbiopolymers. The hydroxyl and ionized sulfate groups makes MTX a highly polar substance, but the presence of large hydrophobic portions make it amphipathic so that it most likely inserts itself deep into the bilayer. MTX elicits Ca^{2+} influx in a variety of cell types, and the Ca^{2+} influx may lead to secondary effects, including phosphinositide breakdown and arachidonic release. Of special interest here in that cells expressing TRPC-1 show a substantial increase in MTX-initiated Ca^{2+} influx that is blocked by Gd^{3+} (K_{D50}=3 μM) and also by amiloride and benzamil but not by flufenamic acid or niflumic acid [10, 14, 174]. MTX activates 40 pS channels when applied to outside-out patches but not inside-out patches indicate that MTX acts on the extracellular face and does not require second messengers [40]. Both the conductance and pharmacological properties have led to the idea that MTX activates the MscCa channels in oocytes, which is consistent with its effect of activating similar channel currents in TRPC-1 expressing cells. On the other hand, MTX also increases Ca^{2+} influx in red blood cell (RBC) ghosts which may involve another mechanism [85]. Although it has been suggested that MTX mainly acts to increase current by stimulating insertion of channels in the oocyte membrane, the evidence is based on large rapid C_m changes that follow moment-to-moment changes in conductance induced by MTX and which are blocked by the same ions and agents that also block the conductance changes [174]. These properties indicate the C_m changes may have been contaminated by changes in membrane conductance [25]. In this case, alternative methods for measuring membrane trafficking (e.g., FMI-43 fluorescence) should be used to test whether MTX-induced

membrane conductance occurs by channel insertion and/or channel activation [40].

Mechanosensitive channel protein identification

The membrane proteins forming specific MS channels have only been recently identified, and there were several reasons for this delay, including the general low abundance of MS channels in animal cells, the absence of high-affinity MS channel agents, the inability to employ conventional expression cloning strategies because of widespread endogenous MS channel expression, and the absence of identified mutant phenotypes involving stretch-activated channels. To overcome these handicaps, a novel strategy was developed by Sukharev et al. [159, 160] and colleagues that involved detergent-solubilizing and fractionating membrane proteins, reconstituting the protein fractions in liposomes, then assaying the fractions for stretch sensitivity using patch clamp recording. This technique has been used to identify a variety of MS channel proteins in bacteria and archaea [81, 103, 158, 159] and, most recently, the TRPC protein family in forming MscCa in *Xenopus* oocytes [97]. Furthermore, by demonstrating that a purified protein reconstituted in pure liposomes can retain stretch sensitivity, the technique also provided unequivocal evidence for the idea that bilayer tension alone gated MS channels [101]. Although ENaC has also been reconstituted in lipid bilayer and reported to show stretch sensitivity, it is not clear whether the proposed mechanism of stretch-induced release of Ca^{2+} channel block operates in situ [75]. At this time, the best evidence for ENaC family members forming MS channels comes from genetic studies of *C. elegans* touch-insensitive mutants [see 8, 39, 47, 62 for reviews].

The proteins forming the other major class of MS channels in animals cells, MscK [79, 118, 150, 168], were identified serendipitously, in that after the first members of the 2 pore domain K^+ (K2P) channel family had already been cloned and shown to form K^+ channels [41, 42, 93], they were subsequently found to be stretch-activated [6, 126, 129]. The recent demonstration that TREK and TRAAK retain stretch sensitivity in CSK-free membrane blebs indicates that they are also bilayer-gated channels [69]. In addition to the MS channel proteins that may function as mechanosensors in situ, there is also an increasing number of voltage-gated and receptor-gated channels as well as peptides that form simple model channels (alamethicin and gramicidin) that display mechanosensitivity [19, 102, 115, 122, 163]. Although these channels may operate on the same general principles that confer mechanosensitivity on membrane proteins, their role, if any, as mechanotransducers remains to be demonstrated.

Mechanosensitive channel dynamics: adaptation/desensitization/inactivation

Gating dynamics (adaptation/inactivation/desensitization) has been shown to play a critical role in the signaling by voltage- and receptor-gated channels and the hair cell mechanotransduction channel [67, 72] and defects in gating dynamics underlie a number of channelopathies [4]. In the initial studies of single MS channel currents, the channels seemed to obey stationary kinetics and were analyzed accordingly [46, 50, 51, 90, 114, 143, 177, 179]. However, with the ability to apply fast pressure steps to the patch [9, 59, 105–108], it became evident that MS channels also displayed dynamics in which the channels either closed reversibly (adaptation or inactivation/desensitization) or faded irreversibly (run down) with constant stimuli [55, 69, 105, 106, 156].

In principle, the reversible closure of MS channels during maintained stimulation can arise through relaxation in either the mechanical force being applied to the channel or the sensitivity to that mechanical force [54, 57] Because mechanical gating arises from the channel protein being sensitive to some mechanical-induced deformation [i.e., either in the bilayer or in CSK/extracellular matrix (ECM) elements], then closure can arise because of a relaxation in the force causing the deformation or a relaxation in the sensitivity to that deformation. For example, in the simplest case of a two-state channel in which the rate constants for channel opening (β) and closing (α) are displacement-sensitive (i.e., for a tethered MS channel) or tension-sensitive (i.e., for bilayer-gated MS channel) the probability of the channel being open (P_o) will be given by:

$$P_o = 1/(1+K)$$

where

$$K = \beta/a$$

Or, in terms of displacement,

$$K = K_0 e^{s(x_0 - x)}$$

where K_0 is the equilibrium constant when the displacement x is equal to the set point x_0 and determines the number of channels open at zero relative displacement, and s is the sensitivity to the relative displacement change (x_0–x). For a bilayer-gated channel, we can substitute displacement with area change. An exponential time relaxation in either s or x_0 can produce the same adapting MS channel currents [57]. Figure 7 illustrates simulations of the stimulus-response relations made, assuming that after a step stimulus, there is an exponential change in either the set point x_0 (Fig. 7a) or the sensitivity factor s (Fig. 7b).

Although both mechanisms predict the same kinetics of channel closure, the consequences on the P_o–X curves are

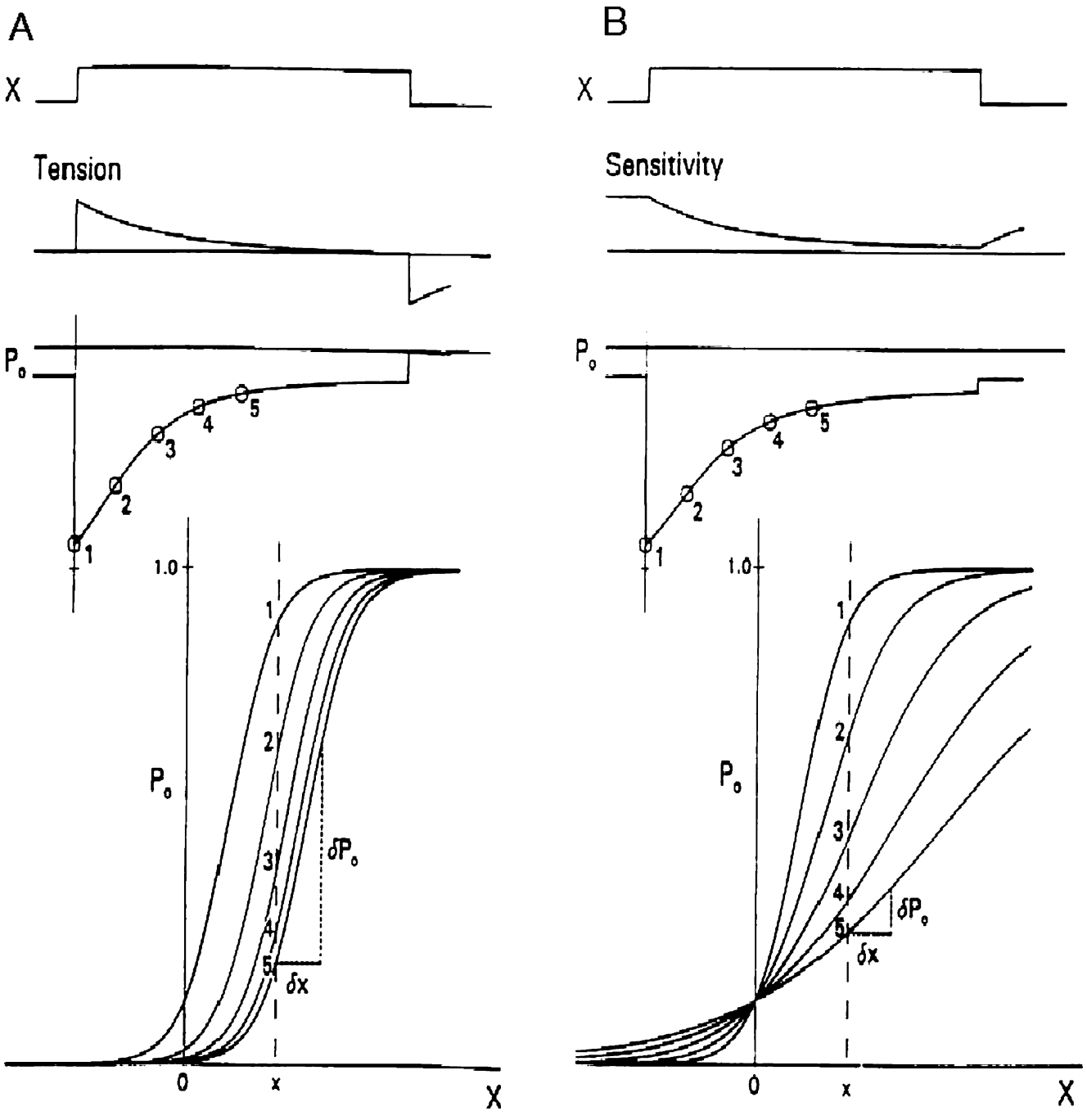

Fig. 7 Simulation of two mechanisms that results in closure of MS channels in the presence of sustained stimulation. A two-state channel is assumed, and a step displacement from 0 to x (*top*, *trace 1*) is used to activate the MS channels (**a** and **b**). *Trace 2* represents the changes in tension (**a**) or sensitivity tension (**b**). In *trace 3*, the channel currents are represented by the change in open-channel probability (P_o), with the *numbered points* (*1–5*) representing equally spaced times where P_o–X curves were generated to follow changes in the MS channel sensitivity. **a** The decay of the current is due to a change in the tension (measured as x–x_0) caused by a shift in the set point. In this case, there is a shift along the x-axis with no change in slope. Consequently, the δP_o response due to δx does not decrease during what can be considered true adaptation. **b** The decay of the current is due to a change in sensitivity in which the slopes of the P_o–X curves decrease as they pivot around a common point. As a consequence, the incremental change in the response (δP_o) for a fixed δx decreases during the current decay, which is akin to receptor desensitization. Modified from Ref. [57]

clearly different. In the first case of adaptation, the curves shift along the x-axis with no changes in slope (i.e., sensitivity) around a common set point. In the second case, the sensitivity decreases as the curves pivot around a common point. From a functional point of view, the first case is true adaptation because sensitivity is maintained

[55], whereas the second case is more akin to receptor desensitization or voltage-gated channel inactivation, where the stimulus must be removed for sensitivity to recover [69]. Below, we consider specific MS channels in terms of these general principles.

MscCa in Xenopus oocytes This channel displays different gating dynamics depending upon patch "history." In the case when the giga-seal is formed using a "gentle" suction protocol (e.g., 10 mmHg for 1–10 s), the application of a suction/pressure step produces rapid opening (<1 ms) of channels followed by a slower closure (~100 ms), although the stimulus is maintained constant. The resultant decay of MscCa current can be fitted by a single exponential with a time constant of around 100 ms at −100 mV that shows a monotonic *e*-fold increase for every approximately 100-mV depolarization. The voltage dependence of the channel closure is most evident when the voltage is switched from hyperpolarized to depolarized potential (or vice versa) during the pressure step (Fig. 8) [55, 107, 108]. The direction of this voltage dependence is similar to the voltage dependence of adaptation displayed by the hair cell mechanotransducer channel [5] and MscS (see below). The stimulus induced closure of the oocyte channel was originally referred to as adaptation because increasing the stimulus could reopen channels. In the oocyte, suctions/pressures of approximately 20 mmHg or more often produce saturating responses (see Fig. 4), so it was assumed that any channel opening caused by an increase in suction/pressure of at least 20 mmHg would involve reopening channels that had just closed. However, a practical limitation in using these protocols on oocyte patches is that application of even larger pulses (e.g., ≥40 mmHg) that would undoubtedly activate all channels will also cause irreversible loss of the channel activity and gating dynamics as described below [55, 105, 106].

In the second case, if a more forceful suction protocol is required to achieve the seal, then more often than not, the transient current response is absent, and instead, the channels remain open for the full duration of the suction. Similarly, if after a gentle seal is formed the patch is mechanically "over-stimulated," then adaptation of MscCa activity disappears either progressively with each moderate-sized pulse (Fig. 9a) or suddenly within a single large suction (Fig. 9b). This transition from the transient mode (TM) to the sustained mode (SM) of gating is irreversible and occurs without a change in single-channel conductance [64].

The fragility of MscCa dynamics and the transition from TM to SM gating has been proposed to arise through mechanical decoupling of CSK interactions with either the channel or the membrane, which are thought to be important for TM of gating. It has been suggested that viscous elements (dashpots) in the CSK can become frozen or decoupled without disconnecting the gating springs. However, adaptation is preserved in both inside-out and outside-out patches, and in patches treated with agents that disrupt microtubules (colchicine) or microfilaments (cytochalasin D), similar to what has been reported for TRAAK desensitization [69]. In contrast, transient gating kinetics of MscCa are not retained in either "blebbed" membrane that it lacks an underlying CSK [185] or in pure liposome patches expressing MscCa activity following reconstitution of detergent-solubilized oocyte membrane proteins [97]. Furthermore, overexpression of TRPC-1 that forms the oocyte MscCa does not result in channel activity that displays TM gating. Whether the absence of adaptation reflects prior mechanodisruption or the absence of CSK remains unclear, as does the mechanism that causes irreversible run down. One possibility is that there are

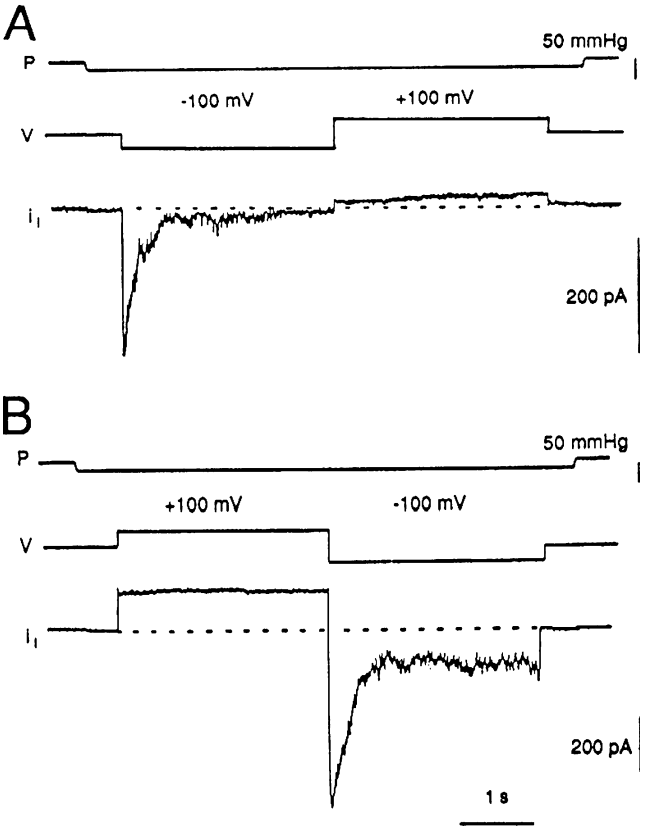

Fig. 8 Voltage dependence of pressure-induced currents recorded from cell-attached oocyte patches. In each *panel*, the *top trace* represents the pressure (suction); the *middle trace*, the voltage; and the *bottom trace*, the current. **a** The application of the suction pulse to the patch held at −100 mV caused rapid opening of channels that had nearly all closed before the voltage was switched to 100 mV while maintaining the suction pulse. **b** In this case, the suction was applied to the patch held 100 mV and produced a steady-state current. Switching the voltage to −100 mV activated a transient increase in current that decayed incompletely in the presence of maintained suction

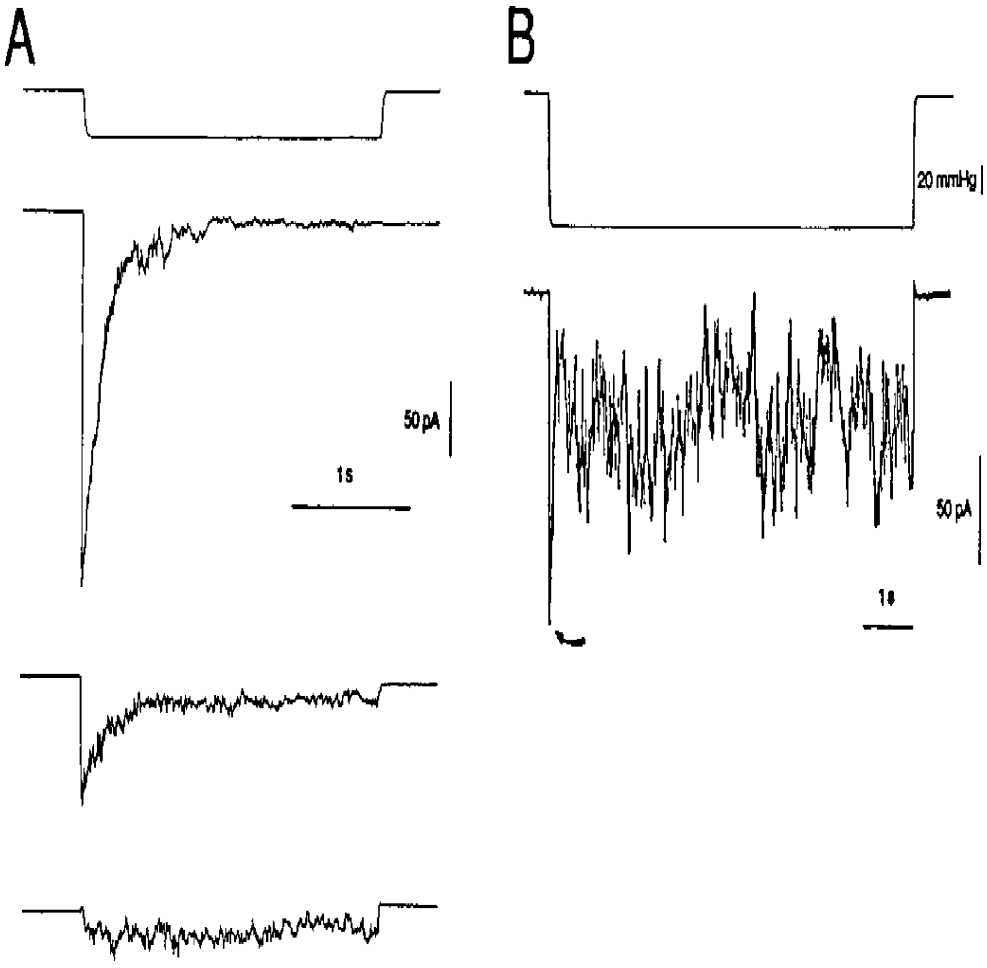

Fig. 9 Irreversible loss of transient mode gating of MscCa. **a** Three consecutive suction pulses of 30 mmHg were applied to a patch 30 s apart, causing a progressive loss of the transient gating and a decrease in the peak current. **b** A single large suction pulse (100 mmHg, 10 s) was applied to a patch and caused an initial peak current that was converted into a sustained current

irreversible changes in the membrane–glass adhesion that alters the ability to generate tension changes in the bilayer. For example, if the membrane does not reseal after mechanical decoupling of lipid–glass interface [121], then increasing pressure may draw further membrane into the pipette without increasing bilayer tension.

MscCa in rat astocytes This channel shows certain dynamic properties similar to those of the oocyte MscCa, including its voltage dependence and mechanical fragility. However, in the astrocyte, the decrease in current occurs because of increased occupancy of lower conductance states and a reduced open-channel probability [13, 156]. Furthermore, the closed channels cannot be reactivated by increasing the stimulus strength (i.e., are refractory), indicating inactivation rather than adaptation. The process was modeled as a ball-and-chain-type inactivation, in which the inactivating ball was a CSK element rather than part of the channel protein. By assuming that the binding rates of the inactivating ball were affected by the position of an intramembrane voltage-sensing subunit, one can account for the voltage dependence of inactivation. Apparently consistent with the model, it was demonstrated that a combination of agents targeting actin (cytochalasin), microtubules (colchicine), and intermediate filaments (acrylamide) was required to abolish the inactivation, but this loss might again reflect general mechanical patch damage rather than implicating specific CSK elements. In the same study, fast C_m measurements were used to monitor the change in membrane area/thickness during pressure steps and demonstrated a similar voltage-independent monotonic increase in patch capacitance at −90 and + 50 mV, which contrasted with the voltage-dependent inactivation. This observation was interpreted as indicating inactivation was due to intrinsic properties of the channel rather than relaxation of bilayer tension [156].

MscS and MscL in Escherichia coli The two predominant MS channels in *Escherichia coli*, MscS (0.5 pS) and MscL (1–3 nS) [100, 160], also exhibit transient gating dynamics [2, 66, 86, 87]. MscS currents measured in *E. coli* protoplasts in response to pressure steps undergo a pressure-induced exponential decay that appeared to be voltage-independent with a time constant of 2–3 s when measured over a narrow voltage range of ±30 mV [86]. However, when measured over a wider voltage range of

±100 mV there was a decrease in the rate of inactivation with depolarization similar to MscCa [2]. Application of the second of a double-step protocol activates fewer channels, and a finite time without stimulation is required for recovery of the full response [86]. Furthermore, suction ramps produced smaller peak responses than suction steps [2]. These features are more consistent with inactivation than adaptation. Unlike MscCa in vertebrate cells, MscS dynamics are not mechanically fragile, although pronase treatment of the intracellular membrane face abolishes the transient kinetics and, ultimately, mechanosensitivity. This last observation led Koprowski and Kubalski to propose that both activation and inactivation may depend upon interaction between a cytoplasmic (pronase-sensitive) region of the channel with the lipid bilayer [86, 87]. Note the proteolytic inhibition of MscS activity is opposite to the potentiation of MscL activity [1]. Given that a bilayer rather than a tethered mechanism gates MscS, it was proposed that inactivation might be associated with insertion of the cytoplasmic domain of MscS in the bilayer (i.e., a "hybrid" or intrinsic tethered model; see below).

MscL reconstituted in liposomes also shows a transient decay in the current with a time constant of seconds [66]. Although the distinction between adaptation and inactivation still needs to be made, the observation is significant because the clear absence of any CSK excludes its involvement in these transient kinetics. One possible explanation is time-dependent sliding/relaxation of the two monolayers that results in relaxation of the gating tension [144].

TREK and TRAAK MS channels In a recent study, pressure steps have been used to analyze the dynamics of MscK formed by cloned TREK-1 and TRAAK herologously expressed in COS cells and *Xenopus* oocytes [69]. Both channels show rapid closure (τ~20–50 ms), with constant stimulation similar to the MscCa. However, unlike MscCa, MscK gating dynamics are not voltage-sensitive, and either mechanical or chemical disruption (i.., using latrunculin) of the CSK causes "run up" rather that "run down" of the channels without removing the transient gating kinetics. Because it was clearly demonstrated that channels could not be reactivated without a finite time for recovery, the phenomenon was referred to as desensitization. The lack

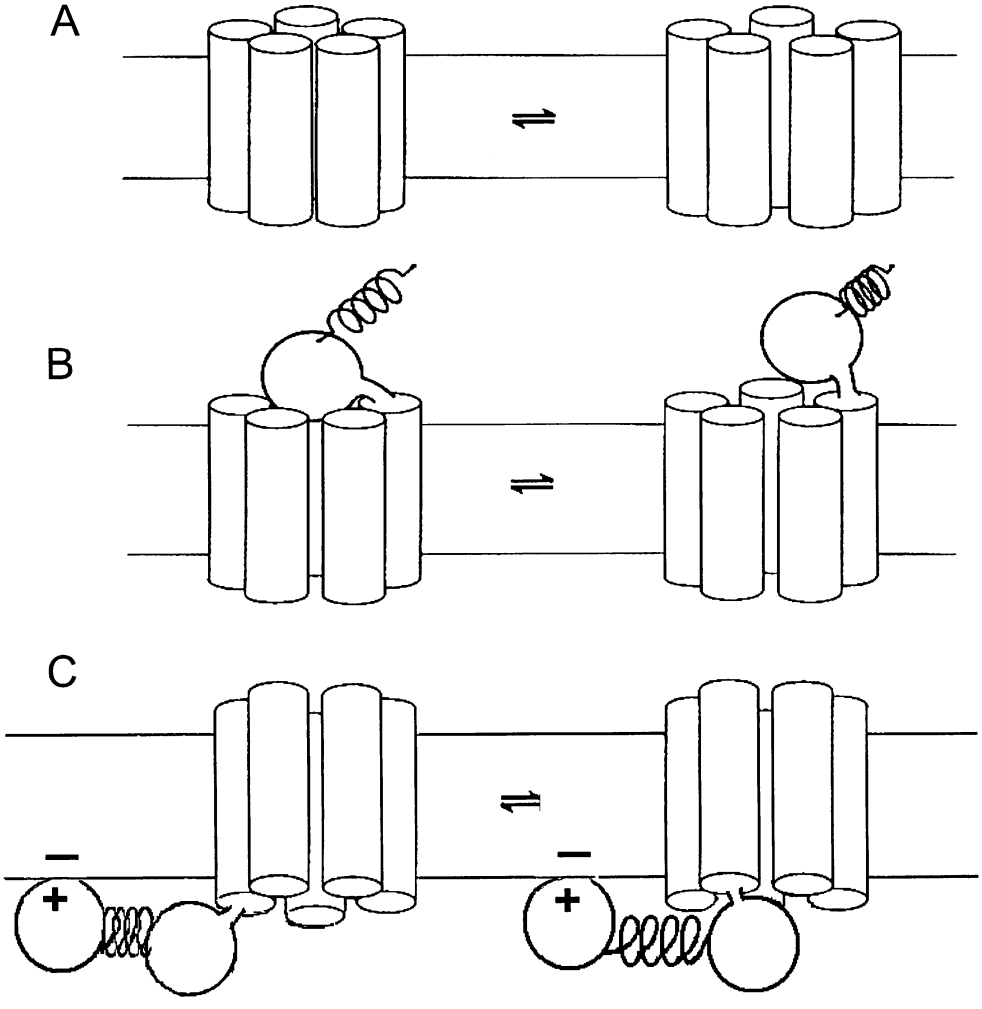

Fig. 10 Three different models of mechanosensitive channel gating **a** Bilayer. Mechanical forces are conveyed to the channel purely via the bilayer. Tension sensitivity occurs because of a difference protein area (or hydrophobic thickness and/or lateral shape) between the open and closed channel conformations. **b** Extrinsic tether. Tensions are exerted directly on the channel protein via extracellular or cytoskeletal elastic elements/gating springs. When tension is exerted on the gating spring, the open state is energetically more favorable. Intrinsic tether (hybrid). In this model, the gating spring is one of the cytoplasmic domains that binds to the phospholipids and, in this way, becomes sensitive to membrane stretch

of effects of either mechanical or chemical CSK disruption indicates that desensitization is an intrinsic property of the channel [69].

Molecular models of stretch sensitivity

There are three broad classes of mechanisms that may impart stretch sensitivity on a membrane ion channel. They will be referred to as "bilayer," "tethered," and "hybrid," and are shown schematically in Fig. 10. The models need not be mutually exclusive, and a single channel may derive its mechanosensitivity from all three mechanisms. Each mechanism can be discussed in terms of a simple two-state channel that fluctuates between a closed and open state. The bilayer mechanism applies to a variety of MS channels, as evidenced by retention of mechanosensitivity following liposome reconstitution and/or activation by amphipaths or lysophospholipids. The basic idea is that stretching the bilayer will tend to decrease its lipid packing density and thickness, so that if the channel protein undergoes a change in membrane-occupied area (Fig. 9a) and/or hydrophobic mismatch, there will be a shift in the distribution between closed and open channel conformations [54, 88, 95]. By inserting in the membrane, lysophospholipids and amphipathic molecules may cause local changes in tension and curvature at the lipid–protein interface and thereby shift the channel distribution [12, 95, 98, 99, 131–133].

In the tethered mechanism, either an extracellular or cytoskeletal protein is directly connected to the channel and acts as a gating spring [50, 58, 62, 71, 72]. When the gating spring is stretched, it favors the open state of the channel because it allows relaxation of the spring. In Fig. 9b, the gate is represented as a trapdoor that opens out, but it can well represent subunits that are either pulled apart (increased in area) or lengthened (change if hydrophobic mismatch). Evidence pro and con for the tethered mechanism has been discussed previously [54, 88].

The hybrid of the above two mechanisms depends upon stretching of the bilayer, but in this case, there are cytoplasmic domains of the channel protein that bind to phospholipids, and in this way act as intrinsic tethers or gating springs that are stretched along with the bilayer (Fig. 9c). Evidence for the hybrid model comes from the identification in the specific K2P channels of a phospholipid-sensing domain on the proximal carboxyl terminus that involves a cluster of positively charged residues that also includes the proton sensor E306 [23, 68]. Protonation of E306 drastically tightens channel–phospholipid interaction and leads to TEK-1 opening at atmospheric pressure. The carboxy terminal domain of TREK-1 interacts with plasma membrane, probably via electrostatic interaction between a cluster of positive charges (a PIP2-interacting domain) and anionic phospholipids.

Mechanosensitive channels in human diseases

An exiting development in the field has been the growing number of diseases associated with abnormalities of mechanotransduction. Donald Ingber [73], in a recent review, listed 45 diseases that may arise due to changes in cell mechanics, alterations in tissue structure, or deregulation of mechanosignaling pathways. Of these diseases, several have been directly associated with changes in expression and/or gating of MS channels, including cardiac arrhythmias [84], polycystic kidney disease [18], hyperten-

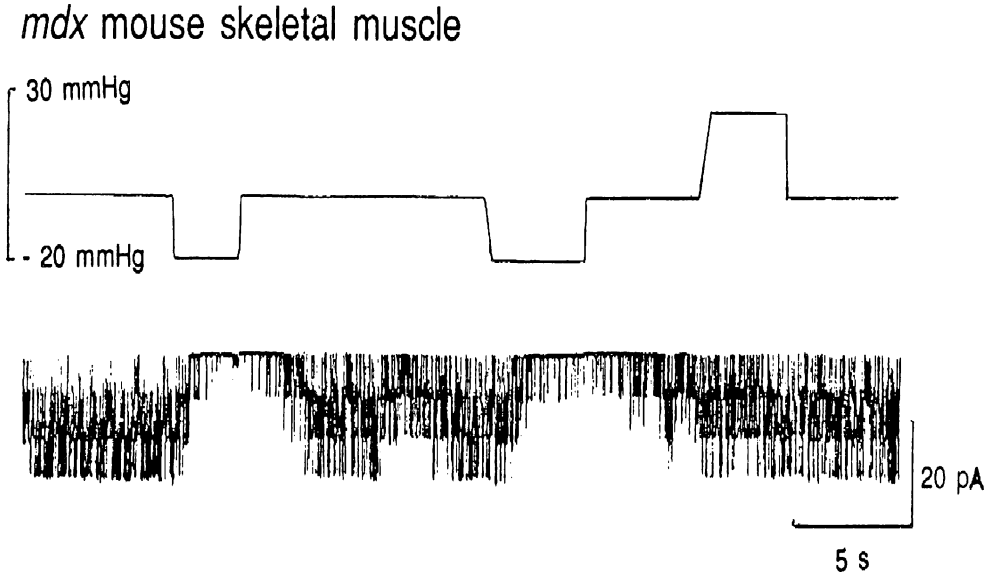

Fig. 11 Cell-attached patch recording on an *mdx* mouse myotube showing high constitutive channel activity that was reduced with suction but unaffected by positive pressure. This "apparent" stretch-inactivated channel activity was rare and seen in only 1 of approximately 100 patches. The majority of other patches showed no spontaneous channel activity, and suction activated either a transient opening of channels or channels that remained open for 10 s after the pulse (e.g., see [107])

sion [83], glioma [123], glaucoma [78] atherosclerosis [22, 134], Duchenne muscular dystrophy [44, 45], and tumorigenesis [128]. Furthermore, increased MscK activity has been shown to prevent brain ischemia [16] and promote general anesthesia [127], whereas MscCa/TRPC activity may regulate wound healing [137] and promote neuronal regeneration [76]. Of particular note is Duchenne muscular dystrophy (DMD), a devastating X-linked genetic disease that affects approximately 1 in 3,500 male births and is characterized by progressive muscle wasting and weakness (reviewed in [180]). DMD is caused by the absence of the gene product of dystrophin, a cytoskeletal protein that binds to actin and provides structural support for the membrane particularly during muscle stretching. In *mdx* muscle fibers (i.e., from the mouse model of DMD), there is increased vulnerability to stretch-induced membrane wounding, and several studies indicate elevated $[Ca^{2+}]_i$ levels in *mdx* myotubes that have been associated with increased Ca^{2+} permeant leak channel activity [43] and/or abnormal MscCa activity [44, 45]. Anti-MscCa agents, including Gd^{3+}, streptomycin, amiloride, and GsMTx-4, have been reported to block Ca^{2+} elevation and/or reduce muscle fiber degeneration [3, 56]. Based on the observation that the leak channel activity was increased by internal calcium store depletion, Vandebrouck et al. [167] proposed that a store-operated Ca^{2+} channel (SOCC) belonging to the TRPC family may be involved. To test this idea, they transfected muscles with antisense oligonucleotide designed against the most conserved region sequences of the TRPCs and showed it caused significant knockdown of TRPC-1 and - 4 but not TRPC-6 (all three were detected in wild-type and *mdx* muscle), and reduced both control and thapsigagin-induced Ca^{2+} leak channels without affecting voltage-gated Na^+ channels. The mechanosensitivity of the channels was not tested in this study. However, MscCa can show significant spontaneous opening in the absence of membrane stretch [140]. Furthermore, although Franco and Lansman [44] initially reported a stretch-inactivated Ca^{2+} channel in *mdx* mice, they subsequently concluded that the channel activity may arise from a novel gating mode of MscCa induced by membrane stress [45]. Most recently, it has been suggested that stretch inactivation in patches of *mdx* muscle and other cells may be a patch recording artifact induced when suction applied to the patch reduces a tonic tension generated by CSK forces that bend the patch toward the cell [69]. At least consistent with this notion is that suction (but not positive pressure) causes inactivation of MscCa in *mdx* patches (Fig. 11).

Conclusion and future prospects

The giga-seal patch clamp technique has been a major contributor to increased understanding of MS channels over the last 20 odd years. However, there is still somewhat a disconnect between the phenomena seen in the patch and how they translate in MS currents in the whole cell. Furthermore, given the growing evidence that MS channels are promiscuous in terms of their modes of activation, it becomes even more important to identify the exact physiological stimulus that activates the channel in specific situations. The development of new techniques that can monitor/generate membrane tension changes in normally operating cells while recording MS channel on the cell can address many of the unresolved issues. Similarly, the discovery of high affinity and selective agents that can target mechanically gated channels will represent a major breakthrough for the field. The determination of the crystal structure of bacterial MS channels [7, 21, 139] has provided a rich environment for model building and testing, and a similar trajectory is predicted for the recently identified MS channel proteins in animal cells. A key question that these studies should answer is whether a unified set of principles can account for the stretch sensitivity of channels in both prokaryotes and eukaryotes [54, 88].

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CHAPTER 17

MscCa Regulation of Tumor Cell Migration and Metastasis

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I. OVERVIEW

The acquisition of cell motility is a required step in order for a cancer cell to migrate from the primary tumor and spread to secondary sites (metastasize). For this reason, blocking tumor cell migration is considered a promising approach for preventing the spread of cancer. However, cancer cells like normal cells can migrate by several different modes referred to as

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"amoeboid," "mesenchymal," and "collective cell." Furthermore, under appropriate conditions a single cell can switch between modes. A consequence of this plasticity is that a tumor cell may be able to avoid the effects of an agent that targets only one mode by switching modes. Therefore, a preferred strategy would be to target mechanisms that are shared by all modes. Here we review the evidence that Ca^{2+} influx via the mechanosensitive Ca^{2+}-permeable channel (MscCa) is a critical regulator of all modes of cell migration and therefore represents a very good therapeutic target to block metastasis.

II. INTRODUCTION

Cancer is a multistep process that results in a normal cell, often an epithelial cell lining a gland, duct, or organ surface, undergoing abnormally increased multiplication to produce a localized primary tumor that with time invades and spreads (metastasizes) to surrounding tissues and eventually causes death. However, in order for a tumor to metastasize, the tumor cell must migrate from the primary tumor, pass through blood vessels, penetrate into the secondary tumor site, and migrate through the tissue to establish a metastasis. Therefore, the acquisition of cell motility is a necessary although not a sufficient step for tumor invasion and metastasis, which also require the additional steps of barrier matrix breakdown, tumor cell adherence, growth, and angiogenesis at the secondary sites. Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in the metastatic cascade, identifying the most sensitive and susceptible step that regulates tumor cell migration should provide a promising target to block metastasis (Grimstad, 1987; Stracke *et al*., 1991; Kassis *et al*., 2001).

There are currently two models used to explain tumor progression to the metastatic disease. One is the traditional "multi-hit" genetic model that proposes a sequence of mutations that triggers the various stages of cancer (e.g., initiation, promotion) with the final mutation(s) promoting increased tumor cell invasiveness and metastasis (Emmelot and Scherer, 1977; Cahill *et al.*, 2000; Hanahan and Weinberg, 2000; Zhou *et al*., 2005). Evidence supporting this model includes the existence of several stable human tumor cell lines that demonstrate high invasiveness when implanted in animals (Kaighn *et al*., 1979; Sung *et al.*, 1998), and the recent discovery that many primary tumor cells already express a genetic signature that predicts their metastatic potential (Ramaswamy *et al*., 2003; Varambally *et al*., 2005). The second model is an epigenetic one based on the discovery that growth factors that trigger the epithelial–mesenchymal transition (EMT), in which nonmotile epithelial cells are converted into motile mesenchymal cells (e.g., during

normal embryogenesis and wound healing), are also released by stromal cells surrounding the tumor and promote increased tumor cell invasiveness and metastasis (Thiery, 2002; Thompson and Newgreen, 2005; but see Tarin, 2005). Specific cancers may utilize one or a combination of the two mechanisms since the mechanisms are not exclusive (e.g., one aspect of the metastatic genetic signature may include the potential to undergo EMT). In any case, the regulatory molecules involved in transforming a tumor cell from a nonmotile to a motile phenotype need to be identified. In this chapter we focus on the role of the MscCa, which is identified as a member of the transient receptor potential channel family (Maroto *et al.*, 2005; Saimi *et al.*, 2007) and shown to be essential for prostate tumor cell migration (Maroto *et al.*, 2007). Because MscCa is expressed by both nonmotile and motile cells, we review the evidence for the idea that changes in MscCa properties triggered by events associated with cancer progression may contribute to increased tumor invasiveness and metastasis.

III. DIFFERENT MODES OF MIGRATION

Normal cells and tumor cells move according to one of three major modes of migration referred to as "amoeboid," "mesenchymal," and "collective cell." Furthermore, under specific circumstances a single cell can switch between these modes (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Friedl, 2004; Wolf and Friedl, 2006). Because of this plasticity, a tumor cell may be able to avoid the effects of an agent that blocks only one migratory mode by switching to another mode. Therefore, a preferred strategy would be to identify and target molecular mechanisms that are shared by all modes. With this in mind, we consider the different modes of migration, their similarities and differences, and in particular their possible common dependence on Ca^{2+} influx via MscCa.

A. Amoeboid Migration

Amoeboid movement is expressed by a variety of invertebrate and vertebrate cells, but has been the most intensely studied in the amoeba *Dictyostelium discoideum.* This cell displays an ellipsoidal profile with either a monopodal or polypodal form, and undergoes a rapid (e.g., >20 μm/min) gliding movement that involves repetitive cycles of protrusion and contraction with little adhesiveness to the substrate. This lack of adhesiveness is consistent with the absence of integrin expression by the amoeba (Friedl, 2004). The amoeba uses two mechanically distinct mechanisms to push itself

forward (Yoshida and Soldati, 2006) a filopodia–lamellipodia mechanism that depends on actin polymerization and a bleb mechanism in which a local region of membrane where the cortical-CSK has been disrupted is pushed outward by cytoplasmic pressure generated by myosin II. Both protrusion mechanisms involve significant mechanical distortions of the membrane at the front of the cell that could activate MscCa to provide feedback (via Ca^{2+} influx and/or membrane polarization) between the force-generating mechanisms and resultant membrane distortions.

Neutrophils, eosinophils, lymphocytes, stem cells, and specific tumor cells associated with leukemia, lymphoma, and small cell lung carcinoma also display amoeboid movement. Furthermore, specific cell types that display a mesenchymal mode of migration when crawling on a two-dimensional (2D) substrate can switch to an amoeboid mode when migrating through a 3D substrate (Friedl, 2004). Vertebrate cells undergoing amoeboid migration also display both blebbing and filopodia–lamellipodia mechanisms of forward protrusion (Sahai and Marshall, 2003; Blaser *et al*., 2006). Fish and amphibian keratocyes may represent a hybrid form of amoeboid/mesenchymal locomotion because they normally show a smooth gliding movement but also express a broad flat lamellipodium. Furthermore, when they become stuck on their substrate they tend to pull out a rear tether and display a more discontinuous ''mesenchymal-like'' locomotion (Lee *et al*., 1999). Interestingly, an amoeba can be induced to develop a broad lamellipodium and undergo keratocyte-like migration by knocking out a gene that regulates the amoeba's aggregation process (Asano *et al*., 2004). However, a double knockout of myosin II and the aggregation gene does not block keratocyte-like migration, indicating that myosin II may be dispensable for this mode of movement.

B. Mesenchymal Migration

Mesenchymal movement is displayed by fibroblasts, neurons, smooth muscle, and endothelial cells, as well as by specific cancer cells from epithelial tumors, gliomas, and sarcomas. In this mode, the cell typically displays a highly polarized morphology with a front lamellipodium, immediately behind which is the lamella, followed by the cell body with the nucleus, and usually ending with a rear tail or tether. Compared with the smooth, gliding amoeboid movement, mesenchymal migration is relatively discontinuous and slower (<1 μm/min) because of its greater adhesiveness and strong dependence on integrin engagement and disengagement from the substrate. Mesenchymal migration can be divided into five steps involving: (1) forward protrusion of the cell's leading edge, (2) formation of adhesions at the front

of the cell with the extracellular matrix (''gripping''), (3) pulling against the ECM via the cell adhesions as the myosin–cytoskeleton (CSK) contracts and exerts traction force against the substrate, (4) progressive stretching of the cell as the traction force develops at the cell front and pulls against the cell rear, and (5) finally, detachment of the rear adhesions from the ECM allowing net cell displacement and relaxation of membrane stretch (Lauffenburger and Horwitz, 1996; Sheetz *et al*., 1999; Ridley *et al*., 2003). The important aspect of this mode of migration in relation to MscCa is that the membrane bilayer of the whole cell will tend to experience a slow ramp of increasing tension for as long as the rate of forward protrusion exceeds the rate or rear retraction (Lee *et al*., 1999; Maroto *et al*., 2007).

C. Collective Cell Migration

In the collective cell mode of migration, the cells are connected by cell junctions formed by cadherins and integrins, and move in a mass with the motile cells at the leading invasive edge generating the adhesion and traction forces (likely via the mesenchymal mode) that tend to pull the rear nonmotile tumor cells along passively. This pattern of migration represents the predominate migration mode for most epithelial cancers *in situ*, and provides the advantage of increased heterogeneity by allowing nonmotile, proliferating cells along with motile path-finding cells to invade the new tissues (Friedl and Wolf, 2003; Wolf and Friedl, 2006).

D. Mechanisms for Switching Migration Modes

Cells that normally express mesenchymal and/or collective cell migration can be converted to the amoeboid mode by reducing the effectiveness of integrin-ECM adhesion (i.e., with integrin-blocking antibodies or arginine-glycine aspartate (RGD) peptides that compete for integrin-ECM-binding sites), by blocking matrix proteases, or by stimulating the Rho-associated serine/threonine kinase (ROCK) that increases cortical contraction, thereby promoting cell rounding and forward protrusion by membrane blebbing (Friedl, 2004). With this switch, the cell becomes more deformable due to its lack of adhesiveness and can now squeeze between matrix barriers. This lessens the dependence on the actions of matrix-degrading metalloproteinases and increases resistance to metalloproteinase inhibitors. The weakened dependence on integrin adhesion also results in a loss of dependence on calpain proteolytic cleavage important for integrin-linked adhesion turnover (Carragher *et al*., 2005). In neutrophils, rear integrins tend to be endocytosed

rather than dissembled by calpain activity, and in contrast to mesenchymal cells, inhibition of calpain actually promotes, rather than inhibits, migration by enhancing cell protrusion and cell spreading (Lokuta *et al.*, 2003). On the other hand, amoeboid movement retains a strong dependence on myosin II contractility as indicated by increased sensitivity to ROCK inhibition (Sahai and Marshall, 2003). Since that both calpain and myosin II are Ca^{2+} sensitive, one would expect that both modes of migration would display Ca^{2+} dependence. Another mechanism that appears to promote mode switching relates to the relocation of cavoelin-1 (Cav-1), a lipid raft-associated protein that colocalizes with MscCa/TRPC1 (Lockwich *et al.*, 2000; Brazier *et al.*, 2003; Maroto *et al.*, 2005). For example, when endothelial cells switch from migration in a 2D to a 3D matrix there is a redistribution of Cav-1, and possibly MscCa, from the back to the front of the cell (Parat *et al.*, 2003). As described below, this shift would be consistent with intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transients being initiated in the front of the amoeboid like neutrophils (Kindzelskii *et al.*, 2004) but in the rear of mesenchymal-like cells (Maroto *et al.*, 2007).

IV. Ca^{2+} DEPENDENCE OF CELL MIGRATION

Although a variety of signaling pathways may regulate cell migration, Ca^{2+} signaling has always been considered a significant player because many of the effector molecules that mediate migration are Ca^{2+} sensitive, including myosin light chain kinase (i.e., that regulates myosin II), calpain, gelsolin, α-actinin, and phosphatase (calcineurin) and integrins (Hendey and Maxfield, 1993; Arora and McCulloch, 1996; Eddy *et al.*, 2000; Mamoune *et al.*, 2003; Franco and Huttenlocher, 2005). The Ca^{2+} regulatory role has been reinforced by the finding that a variety of Ca^{2+} transport proteins including pumps, exchangers, and various gated Ca^{2+} channels can modulate cell migration (Dreval *et al.*, 2005).

A. Measuring $[Ca^{2+}]_i$

The most convenient and common method used to measure $[Ca^{2+}]_i$ involves using fluorescent microscopy and Ca^{2+}-sensitive fluorescent dyes like fura-2 and its membrane permeable form fura-2 AM (Grynkiewicz *et al.*, 1985). The main advantage of the approach is that changes in $[Ca^{2+}]_i$ can be monitored while simultaneously measuring cell migration (i.e., by time-lapse videomicrosopy). As a consequence, one can relate specific spatio-temporal changes in $[Ca^{2+}]_i$ to specific events occurring during migration. However,

there are also some practical limitations associated with the method, including the difficulty of detecting local vs global $[Ca^{2+}]_i$ changes and the possibility of compartmentalization of the dyes in organelles. The first limitation has been somewhat overcome by recent technical developments that includes the use of total internal reflectance fluorescence microscopy that offers added spatial resolution to allow detection of single-channel $[Ca^{2+}]_i$ fluctuations at the ventral membrane surface adhering with the glass surface (Demuro and Parker, 2005). In addition, the development of Ca^{2+}-sensor "cameleons" that operate by fluorescence energy transfer and can be targeted to the plasma membrane or the ER can be used to measure $[Ca^{2+}]_i$ changes in these membrane microdomains (Miyawaki *et al*., 1997; Isshiki *et al*., 2002). In the case of fura-2 compartmentalization, there are discrepant views on its occurrence and significance. For example, one group has proposed that the apparent $[Ca^{2+}]_i$ gradient seen in T lymphocytes is due to fura-2 accumulation in mitochondria (Quintana and Hoth, 2004), whereas another group found that the $[Ca^{2+}]_i$ gradient seen in fibroblasts was not associated with mitochondria but instead colocalized with the Golgi apparatus in the perinuclear region (Wahl *et al*., 1992). A further complication is that mitochondria are motile, and their motility varies inversely with $[Ca^{2+}]_i$ so that they move fastest in lower $[Ca^{2+}]_i$ (100-300 nM) but stop movement in higher $[Ca^{2+}]_i$ (i.e., 1 μM) (Yi *et al*., 2004). As a consequence, one would expect mitochondria to migrate up a $[Ca^{2+}]_i$ gradient and accumulate in regions of highest $[Ca^{2+}]_i$ where they may function as Ca^{2+} buffers and/or prevent the spread of local $[Ca^{2+}]_i$ transients (Tinel *et al*., 1999; Yi *et al*., 2004; Levina and Lew, 2006). However, in apparent contradiction of this idea, mitochondria accumulate in the lamellipodium of migrating fibroblasts and prostate tumor cells (DeBiasio *et al*., 1987; Maroto *et al*., 2007), and yet these cells develop a global $[Ca^{2+}]_i$ gradient that increases from front to back of the cell (Hahn *et al*., 1992; Matoto *et al*., 2007). The stimulus that promotes this accumulation remains unclear but could involve the added requirement for ATP and/or an elevated $[Ca^{2+}]_i$ in membrane subdomains of the lamellipodium. In any case, it would appear that compartmentalization of fura-2 dye cannot alone explain the sustained, and in some cases rapidly reversible, $[Ca^{2+}]_i$ gradients seen in a variety of migrating cells (see Section IV.E.2).

B. Identifying Ca^{2+} Influx Pathways

The simplest method to demonstrate a requirement for Ca^{2+} influx is to show that migration requires the presence of external Ca^{2+} (Strohmeier and Bereiter-Hahn, 1984). Patch clamp recording can then be used to characterize the kinetics, conductance, surface distribution, and pharmacological

properties of the Ca^{2+} channels expressed in the migrating cell (Lee *et al*., 1999; Maroto *et al*., 2007). With this knowledge one can then use various treatments to relate particular $[Ca^{2+}]_i$ changes to specific Ca^{2+} channels activities. One perceived practical limitation of patch clamping is that channel current measurements are restricted to the dorsal surface because it is not possible to patch the ventral "adherent" surface, at least with the traditional patch clamp method (Hamill *et al*., 1981). In this case, one might argue that because CSK-generated mechanical (traction) forces are transmitted to the substrate purely at ventral surface adhesions, then only mechanosensitive processes in these sites will experience mechanical force and become activated (Mobasheri *et al*., 2002). However, the traction forces that pull on the substrate via the ventral surface adhesions will also tend to stretch the whole cell for as long as the rear of the cell remains firmly attached to the substrate. Apart from causing the cell to become extended, there are other manifestations of these stretching forces including the smoothing out of membrane folds and microvilli in spreading cells (Erickson and Trinkhaus, 1976), an elastic recoil seen occasionally in some migrating cells as presumably stretching forces exceed adhesive forces (Mandeville and Maxfield, 1997), and even cell rupture/fragmentation that can occur when cell retraction is blocked and the pulling forces exceed the elastic limits of the bilayer (Verkhovsky *et al*., 1989). Galbraith and Sheetz (1999) have elegantly and directly addressed the issue of force distribution on the ventral and dorsal surfaces by using optical tweezers to measure the membrane tension on the dorsal membrane, and a micromachined device to measure tension generated on the ventral membrane. Their measurements indicate that the dorsal matrix is as effectively linked to the force-generating CSK as the ventral adhesions so tension-sensitive channels located in both the dorsal and ventral surfaces should experience the same stretch. In this case, the MscCa properties measured on the dorsal surface (i.e., their gating kinetics and subsurface distribution) should be important in defining the $[Ca^{2+}]_i$ dynamics measured during cell migration (Maroto *et al*., 2007).

C. Ca^{2+} Dependence of Amoeba Locomotion

One of the earliest observations implicating Ca^{2+} in amoeboid migration was that lanthanum, a known Ca^{2+} channel inhibitor, blocked movement of *Amoeba discoides* (Hawkes and Hoberton, 1973). Subsequently, microinjection of aequorin (a photoprotein that emits light on Ca^{2+} binding) was used to demonstrate a sustained $[Ca^{2+}]_i$ elevation in the tail of the amoeba, as well as transient Ca^{2+} influxes in the tips of advancing pseudopods—lowering external $[Ca^{2+}]_o$ did not immediately reduce rear $[Ca^{2+}]_i$ but it did block

continued migration (Taylor *et al*., 1980). This was interpreted as indicating that rear $[Ca^{2+}]_i$ can be maintained by Ca^{2+} release from internal stores, but migration is more sensitive to Ca^{2+} influx into the pseudopod tips (Taylor *et al*., 1980). In another study, direct injection of fura-2 was used to show that monopodal amoebae developed a continuous $[Ca^{2+}]_i$ gradient increasing from front to rear, whereas polypodal amoebae showed a decrease in $[Ca^{2+}]_i$ in extending pseudopodia, and an increase in retracting pseudopodia (Gollnick *et al*., 1991; Yumura *et al*., 1996). Subsequently, intracellular BAPTA, a fast Ca^{2+} buffer, was shown to reduce cell spreading, pseudopodia formation, and amoebae locomotion, and these effects could be reversed by raising $[Ca^{2+}]_o$ (Unterweger and Schlatterer, 1995). On the other hand, the same study found that chelation of $[Ca^{2+}]_o$ by the relatively slow Ca^{2+} buffer EGTA did not block pseudopod formation, although it did block the development of any $[Ca^{2+}]_i$ gradient and cell migration. Nebl and Fischer (1997) used recombinant aequorin to demonstrate that chemoattractants induced an increase in $[Ca^{2+}]_i$ that was entirely dependent on Ca^{2+} influx, and speculated that Ca^{2+}-induced actin depolymerization in the rear acted to prevent the formation of stable pseudopod formation in this region of the cell. $[Ca^{2+}]_o$ was shown to be required for shear-flow-induced amoebae motility (but not directionality) and that addition of either EGTA or Gd^{3+} stopped cell movement (Fache *et al*., 2005). In this case, the effects of external Ca^{2+} were shown to stimulate cell speed by increasing the amplitude, but not the frequency, of both protrusion and retraction events at the cell's leading edge (Fache *et al*., 2005). Another study based on mutants lacking two major Ca^{2+}-binding proteins in the ER (calreticulum and calnexin) concluded that chemotaxis depended on both Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release from internal stores (Fisher and Wilczynska, 2006).

Despite the above results, there are also several studies that seem to discount a critical role for Ca^{2+} in amoeboid migration. For example, based on normal chemotaxis seen in a mutant amoeba lacking an IP_3-like receptor, it was concluded that Ca^{2+} signaling was not required for chemotaxis (Traynor *et al*., 2000). However, different groups studying the same mutant found that $[Ca^{2+}]_i$ transients dependent on Ca^{2+} influx were not only retained but were required for both chemotaxis and electrotaxis (Schaloske *et al*., 2005; Shanley *et al*., 2006). In a different study, it was reported that amoebae can continue their random locomotion with the same speed in the absence of $[Ca^{2+}]_o$ and the presence of 50-mM EGTA or EDTA, apparently ruling out any role for Ca^{2+} influx (Korohoda *et al*., 2002). However, a more trivial explanation may relate to inadvertent Ca^{2+} leaching from the low profile glass chamber in which both the ventral and dorsal surfaces of the migrating cell make close contact with the glass. Under these conditions, Ca^{2+} may build up in the narrow gaps between the adherent cell and glass

surfaces and reach levels (~1 μM) sufficient to support migration (Fisher and Wilczynska, 2006). A similar phenomenon may also account for the apparent lack of external Ca^{2+} dependence of human leukocyte locomotion when they are "chimneying" between closely apposed glass slide and cover slip (Malawista and Boisfleury-Chevance, 1997).

In summary, while most studies indicate that both Ca^{2+} influx and $[Ca^{2+}]_i$ elevations are required for an amoeba to migrate, the exact role of Ca^{2+} influx in forward protrusion and rear retraction needs to be better defined. There also remains the unresolved issue on whether the reports of amoeba's migration in the absence of $[Ca^{2+}]_o$ are real or artifactual. In particular, it will be interesting to test whether migration by chimneying is retained in the presence of the faster Ca^{2+}-buffering capacity of BAPTA.

D. Ca^{2+} Dependence of Vertebrate Cell Amoeboid Migration

Newt neutrophils, which are relatively large (~100 μm in diameter) and comparable in size to an amoeba, develop a sustained $[Ca^{2+}]_i$ gradient that increases from front to rear of the cell as they migrate. Furthermore, spontaneous changes in $[Ca^{2+}]_i$ gradient direction result in changes in migration direction (Brundage *et al*., 1991; Gilbert *et al*., 1994). In contrast, the smaller human neutrophils do not develop a detectable $[Ca^{2+}]_i$ gradient but instead display $[Ca^{2+}]_i$ transients when migrating on adhesive substrates (e.g., polylysine, fibronectin, or vitronectin), but not on nonadhesive substrates (Marks and Maxfield, 1990; Hendey and Maxfield, 1993). These $[Ca^{2+}]_i$ transients can be blocked, along with neutrophil migration, by either removing $[Ca^{2+}]_o$ or buffering $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$-buffered neutrophils apparently become immobilized because they are unable to retract their rear, which remains anchored to the adhesive substrate. However, they are still capable of spreading, assuming a polarized morphology, and extending their plasma membrane. Furthermore, their motility can be restored by using RGD peptides to block specific integrin attachments to the substrate. Since a similar block of motility could be induced by inhibitors of the Ca^{2+}-dependent phosphatase, calcineurin, it was proposed that this enzyme mediated Ca^{2+}-dependent detachment of the integrin–substrate adhesions (Hendey and Maxfield, 1993). However, the same group latter suggested that a more general mechanism for rear detachment may involve Ca^{2+}-increased myosin II contractility (Eddy *et al*., 2000). A similar Ca^{2+} and RGD sensitivity was seen for neutrophils migrating through a 3D matrigel substrate (Mandeville and Maxfield, 1997), whereas neutrophils migrating on nonadhesive substrates (e.g., glass in the presence of albumin/serum or through cellulose filters) did not display Ca^{2+} transients nor did they require the presence of

external Ca^{2+} or elevations in $[Ca^{2+}]_i$ in order to migrate (Zigmond *et al.*, 1988; Marks and Maxfield, 1990; Hendey and Maxfield, 1993; Laffafian and Hallet, 1995; Alterafi and Zhelev, 1997). A similar phenomena may occur in the normally gliding fish keratocytes that show an increased frequency of $[Ca^{2+}]_i$ transients when their rear becomes transiently stuck on the substrate (Lee *et al.*, 1999). An apparently different role for Ca^{2+} signaling involves Ca^{2+} influx-mediated "priming" of nonmotile eosinophils that enables them to undergo transepithelial migration. However, once the cells are primed, they can migrate in the absence of $[Ca^{2+}]_o$, although they still depend on $[Ca^{2+}]_i$ elevations (Liu *et al.*, 1999, 2003).

In summary, some of the discrepancies in the Ca^{2+} dependence of neutrophil migration may arise through differences in substrate adhesiveness with the strongest Ca^{2+} dependence seen on sticky substrates, but little or no Ca^{2+} dependence on nonadhesive substrates. At least in this respect, vertebrate cells that display the amoeboid mode may differ from the amoeba, which retains Ca^{2+} dependence even though the amoeba does not depend on integrin adhesion. At least for human neutrophils, $[Ca^{2+}]_i$ transients rather than gradients appear to be more important in regulating cell migration by promoting rear retraction possibly by increased adhesion disassembly via increases in calcineurin, MLCK, and/or calpain activity.

E. The Role of $[Ca^{2+}]_i$ Gradients and Transients in Mesenchymal Cell Migration

Cells migrating in the mesenchymal mode can also display sustained $[Ca^{2+}]_i$ gradients and/or fast transients. Since these different spatio-temporal $[Ca^{2+}]_i$ dynamics may regulate different steps associated with the mesenchymal migratory cycle, they will be discussed separately below.

1. A Model for Sustained $[Ca^{2+}]_i$ Gradients

A basic question from the onset is how any cell can maintain a sustained $[Ca^{2+}]_i$ gradient for as long as several hours in a cytoplasm that allows free diffusion of Ca^{2+}. In particular, the existence of any stable regions of different $[Ca^{2+}]_i$ within a continuous aqueous medium would seem to disobey the second law of thermodynamics according to which solutes should passively diffuse down their concentration gradient until they reach equilibrium—in the case of Ca^{2+}, this equilibration should occur in seconds or at most minutes. To explain this apparent paradox, Braiman and Priel (2001) proposed that the cell uses energy to actively take up Ca^{2+} uptake into internal stores that can then be passively allowed to leak out into localized regions of the cytoplasm. By this process, combined with a polarized distribution of Ca^{2+} release

channels on a contiguous ER Ca^{2+} store, the cell could create a sustained $[Ca^{2+}]_i$ elevation in specified subdomains of the cell (Petersen *et al.*, 2001). The interesting aspect of this model is that, one could have uniform Ca^{2+} influx across the cell surface and uniform active uptake by the internal Ca^{2+} stores as long as there was a gradient of Ca^{2+} release from the stores. A further prediction of this model is that if both active uptake and passive leak occur in very close proximity of the membrane, then a subcortical membrane domain of elevated $[Ca^{2+}]_i$ could be maintained that might go undetected by techniques that only measure global $[Ca^{2+}]_i$.

2. $[Ca^{2+}]_i$ Gradients Determine Migrational Directionality

In several cells undergoing mesenchymal migration, $[Ca^{2+}]_i$ gradients have been shown to be important in determining migration directionality. In particular, Xu *et al.* (2004) observed that migrating cerebellar granule cells develop a $[Ca^{2+}]_i$ gradient (low front–high back) according to their migration direction. Furthermore, experimental reversal of the $[Ca^{2+}]_i$ gradient by the application to the front of the cell, an external gradient of various agents that cause $[Ca^{2+}]_i$ elevation (e.g., chemo-repellant slit2, acetylcholine, and ryanodine) was found to be accompanied by a reversal in migration direction. Similarly, if an external gradient of BAPTA-AM was applied to the back of the cell, again the $[Ca^{2+}]_i$ gradient and migration direction was reversed. Although some of the same neurons also displayed occasional $[Ca^{2+}]_i$ transients, no causal relationship was noted between the transients and migration direction (Xu *et al.*, 2004). Similar $[Ca^{2+}]_i$ gradients related to migration direction have been seen in migrating fibroblasts, kidney epithelial tumor cells, vascular endothelial cells, and prostate tumor cells (Hahn *et al.*, 1992; Schwab *et al.*, 1997; Kimura *et al.*, 2001; Maroto *et al.*, 2007). Moreover, Schwab and colleagues have proposed that the relatively high Ca^{2+}-activated K^+ activity evident in the rear of migrating kidney epithelial tumor cells was a direct consequence of a $[Ca^{2+}]_i$ gradient rather than polarized surface expression of the K^+ channels (Schwab *et al.*, 1995, 2006). They also proposed that the underlying basis for the $[Ca^{2+}]_i$ gradient was due to a combination of higher density of Ca^{2+} influx pathways and ER $[Ca^{2+}]_i$ stores in the cell body compared with the lamellipodia (Schwab *et al.*, 1997). Studies of the highly motile prostate tumor cell line, PC-3, have confirmed some of these ideas (Maroto *et al.*, 2007).

$[Ca^{2+}]_i$ gradients are seen not only in migrating cells but also in polarized exocrine acinar gland cells where they may regulate unidirectional fluid secretion. In particular, a time-dependent reversal of the $[Ca^{2+}]_i$ gradient from the luminal to blood side of the acinar cell after acetylcholine (ACh) stimulation has been proposed to be the main basis for a push-pull model for unidirectional fluid secretion (Kasai and Augustine, 1990). In this model,

$[Ca^{2+}]_i$ elevation, first on the luminal cytoplasmic side of the cell causes Cl^- and water efflux into the lumen, then $[Ca^{2+}]_i$ elevation on the blood side of the cell causes Cl^- and water influx from the blood side. Although both cell surfaces express the same Ca^{2+}-activated Cl^- channel, the depolarization that follows ACh stimulation shifts the Cl^- driving force from efflux to influx. A somewhat similar mechanism could presumably underlie the role of ion and water movements in coordinating cell locomotion (Schwab *et al.*, 2006). This possibility seems to be reinforced by the demonstration that aquaporins are selectively expressed in the leading edge of migrating cells (Saadoun *et al.*, 2005). A quite different cell function related to a sustained $[Ca^{2+}]_i$ gradient involves tip growth of fungi in which elevated $[Ca^{2+}]_i$ in the growing tip has been proposed to promote increased insertion of new membrane via exocytosis (Silverman-Gavrila and Lew, 2003). This mechanism would seem unlikely to account for migration directionally since exocytosis predominates at the cell front while endocytosis occurs mainly at the cell rear (Bretscher and Aguado-Velasco, 1998). A more plausible effect of the $[Ca^{2+}]_i$ gradient in promoting cell migration would be to induce polarization of the activities of enzymes regulating actin polymerization/depolymerization, integrin activation/assembly/disassembly, and myosin II contractility (Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1999; Ridley *et al.*, 2003).

3. $[Ca^{2+}]_i$ Transients

$[Ca^{2+}]_i$ transients have been associated with an even wider variety of other processes including fertilization, cell differentiation, exocytosis, muscle contraction, phagocytosis, and neuronal outgrowth and migration (Berridge *et al.*, 2003). This may be because a $[Ca^{2+}]_i$ transient provides a more efficient and safe way to achieve high levels of $[Ca^{2+}]_i$ compared with steady-state elevations. Furthermore, the temporal component of the signal provides an added dimension in terms of encoding information. $[Ca^{2+}]_i$ transients can take a number of forms in motile cells—they can be highly localized and associated with pseudopod (or bleb) protrusion or retraction, they can spread throughout the cell as a regenerative $[Ca^{2+}]_i$ wave, or they can circumnavigate the perimeter of a cell in a clockwise or anticlockwise direction (Kindzelskii *et al.*, 2004). $[Ca^{2+}]_i$ transients can be generated spontaneously or can be induced experimentally by electrical, chemical, and mechanical stimuli. In particular, it has been shown that direct mechanical stretch of fibroblasts and keratocytes, and osmotic swelling of endothelial cells can induce $[Ca^{2+}]_i$ transients (Arora *et al.*, 1994; Oike *et al.*, 1994; Lee *et al.*, 1999; Wu *et al.*, 1999). $[Ca^{2+}]_i$ transients may also have different initiation sites on different cells and these site may vary within a single cell during the course of the migratory cycle. In particular, the initiation sites of

$[Ca^{2+}]$ transients have been related to the distribution of membrane rafts and caveolae (i.e., invaginated membrane structures), which contain the molecular signaling machinery required for Ca^{2+} signaling, and can undergo redistribution during migration and specific forms of stimulation. Membrane raft- and caveolae-dependent Ca^{2+} signaling has been observed in cells undergoing both mesenchymal migration (Manes *et al*., 1999; Isshiki *et al*., 2002; Parat *et al*., 2003; Rizzo *et al*., 2003) and amoeboid migration (Gomez-Mouton *et al*., 2001; Pierini *et al*., 2003; Kindzelskii *et al*., 2004). For example, Isshiki *et al.* (2002) found that the caveolae in quiescent endothelial cells are clustered around the edge of the cell but when stimulated to migrate, either by wounding a cell monolayer or by exposing the cells to laminar shear stress, the caveolae move to the trailing edge of the cell, concomitant with this relocation the sites of Ca^{2+} waves initiation move to the same location (see also Rizzo *et al*., 2003; Beardsley *et al*., 2005). In contrast, in human neutrophils lipid rafts and $[Ca^{2+}]_i$ transient initiation sites have been localized to the leading edge of the migrating cells, and cholesterol depletion, which disrupts raft structure, was found to block both $[Ca^{2+}]_i$ transient initiation and cell migration (Manes *et al*., 1999; Kindzelskii *et al*., 2004). Some insight into the different results may be related to the demonstration that both the leading edge and rear of lymphocytes are enriched in lipid components that partition into different raft-like domains (Gomez-Mouton *et al*., 2001) and that Cav-1, a raft maker, shows a different polarized distribution in endothelial cells depending on whether the cells were migrating on 2D substrate or through a 3D matrix (Parat *et al*., 2003). In particular, Cave-1 moves from the cell's rear to the cell's front during the switch from the 2D/mesenchymal to the 3D/amoeboid migration modes. These findings are highly intriguing giving that TRPC1, a structural subunit of MscCa (Maroto *et al*., 2005), colocalizes with Cave-1-associated membrane lipid rafts (Lockwich *et al*., 2000; Brazier *et al*., 2003) and has been localized at the leading edge of migrating neutrophils (Kindzelskii *et al*., 2004) and the rear of migrating prostate tumor cells (Maroto *et al*., 2007). Together these results indicate that MscCa may redistribute to different regions of the cell surface and perform different, yet critical functions depending on the mode of migration. In this case, MscCa seems to meet the critical criterion of modulating all modes of migration, and unlike integrins, myosin II, calpain, and metalloproteases should not become dispensable following a switch in migration mode.

4. $[Ca^{2+}]_i$ Transients Promote Cell Migration but Inhibit Neurite Outgrowth

$[Ca^{2+}]_i$ transients have been positively correlated with cell migration in cerebellar granular cells, neutrophils, vascular smooth muscle, keratocytes and astrocytoma cells (Komuro and Rakic, 1996; Lee *et al*., 1999; Ronde

et al., 2000; Scherberich *et al.*, 2000; Giannone *et al.*, 2002). Furthermore, the cessation of $[Ca^{2+}]_i$ transients has been correlated with the termination of granule cell migration (Kumuda and Komuro, 2004). In contrast, high-frequency $[Ca^{2+}]_i$ transients cause nerve growth cone stalling and axon retraction, while the inhibition of $[Ca^{2+}]_i$ transients stimulates the extension of axonal growth cones and the outgrowth of axonal and dendritic filipodia (Gomez and Spitzer, 1999; Gomez *et al.*, 2001; Robles *et al.*, 2003; Lohmann *et al.*, 2005). The $[Ca^{2+}]_i$ transients in all cases appear to depend on MscCa-mediated Ca^{2+} influx because they are blocked by anti-MscCa agents (Lee *et al.*, 1999; Jacques-Fricke *et al.*, 2006). Furthermore, the opposite effects both appear to depend on calpain activity (Huttenlocher *et al.*, 1997; Robles *et al.*, 2003). However, whereas calpain activity in the cell rear acts to cleave integrin–CSK linkages and in this way promotes rear retraction and cell migration (Huttenlocher *et al.*, 1997), calpain activity in the nerve growth cone and filopodia acts by promoting actin–integrin disengagement at the front of the process, thereby reducing the traction forces required for lamellar protrusion and growth cone translocation (Robles *et al.*, 2003). Interestingly, calpain inhibition in resting neutrophils promotes polarization and random migration whereas it reduces the neutrophil's capacity for directional migration toward chemotactic stimuli (Lokuta *et al.*, 2003). This may occur because constitutive calpain activity in resting neutrophils acts as a negative regulator of polarization and migration, whereas the polarized calpain activity in chemotaxing neutrophils promotes directional persistence in a chemo-attractant gradient.

V. THE ROLE OF MscCa IN CELL MIGRATION

A key issue for all modes of cell migration is the nature of the mechanosensitive molecules that act to coordinate forward cell protrusion with rear cell retraction. An attractive candidate is MscCa that because of its unique ability to transduce membrane stretch/cell extension and transduce this into a Ca^{2+} influx (Guharay and Sachs, 1984; Sachs and Morris, 1998; Hamill and Martinac, 2001; Hamill, 2006) can provide feedback between mechanical forces that tend to extend the cell and the Ca^{2+}-sensitive regulators of force generation and cell–substrate adhesion. The first indirect evidence for a role of MscCa in cell migration was provided by the demonstration that the nonspecific MscCa blocker Gd^{3+} (Yang and Sachs, 1989; Hamill and McBride, 1996) blocked fish keratocyte migration (Lee *et al.*, 1999; Doyle and Lee, 2004; Doyle *et al.*, 2004). Subsequent studies, also using Gd^{3+}, further implicated MscCa in migration of a mouse fibroblast cell line, NIH3T3 (Munevar *et al.*, 2004), and the human fibrosarcoma cell line, HT1080 (Huang *et al.*, 2004).

However, these studies indicated different sites (i.e., front or back) and different actions (i.e., rear retraction, development of tractions forces, and disassembly of focal adhesions) for MscCa mediated Ca^{2+} influx, which may partly depend upon different modes of cell migration. Significant limitations in these early studies were the lack of protein identity of MscCa and the absence of MscCa-specific reagents, which have been overcome by the recent identification of the canonical transient receptor potential (TRPC1) (Wes *et al.*, 1995) as an MscCa subunit (Maroto *et al.*, 2005), and the discovery of a highly selective MS channel blocker, GsMTx4 a peptide isolated from the tarantula (*Grammostola spatulata*) venom (Suchyna *et al.*, 2004). Several studies have already implicated TRPC1 in regulating cell migration. For example, Huang *et al.* (2003) showed immunohistologically that TRPC1 was expressed in a punctuate pattern around the cell periphery, and based on Gd^{3+} block proposed that TRPC1 supported $[Ca^{2+}]_i$ transients and cell migration. Rao *et al.* (2006) while studying an intestinal epithelial cell line demonstrated that suppression of TRPC1 inhibited cell migration, whereas TRPC1 overexpression of TRPC1 enhanced cell migration as measured by an *in vitro* wound closure assay. Maroto *et al.* (2007) characterized MscCa in both motile (PC-3) and nonmotile (LNCaP) human prostate tumor cell lines and found that MscCa displayed the same single-channel conductance, Mg^{2+} and Gd^{3+} sensitivity as the MscCa endogenously expressed in *Xenopus* oocytes identified as formed by TRPC1 (Maroto *et al.*, 2005). Furthermore, MscCa activity was shown to be required for cell migration based on the block by anti-MscCa/TRPC1 agents including GsMTx4, an anti-TRPC1 antibody raised against the external pore region of the channel, siRNA suppression, and overexpression of TRPC1.

Apart from MscCa, there are other Ca^{2+} channels that have been implicated in regulating cell migration including both the T-type (Huang *et al.*, 2004) and L-type voltage-gated Ca^{2+} channels (Yang and Huang, 2005) that may also display mechanosensitivity (Morris and Juranka, Chapter 11, this volume). Also in addition to the TRPCs, which have been implicated in forming MscCa, other TRP subfamily members are expressed in tumor cells and have been implicated in different steps associated with cancer (Peng *et al.*, 2001; Wissenbach *et al.*, 2001; Nilius *et al.*, 2005; Sánchez *et al.*, 2005). Of particular interest is TRPM7 that has been shown to regulate cell adhesion by regulating calpain via Ca^{2+} influx through the channel (Su *et al.*, 2006) and actomyosin contractility via intrinsic kinase activity of TRPM7 (Clark *et al.*, 2006). Although TRPM7 stretch sensitivity has not been directly demonstrated, it has been shown that fluid shear stress-applied human kidney epithelial cells promote membrane trafficking of TRPM7 to the cell surface (Oancea *et al.*, 2006). Given that fluid shear stress can also trigger cell migration (Isshiki *et al.*, 2002), this may provide an additional MS mechanism to regulate cell motility. In this case, it will be interesting to

determine whether the shear-induced increase in TRPM7 surface expression is also dependent on specific integrin engagement (Maroto and Hamill, 2001) and/or related to the flow-induced recruitment of caveolae to specific regions of the migrating cell (Rizzo *et al*., 2003; Navarro *et al*., 2004).

There are other classes of gated channels that have been implicated in regulating cell migration including voltage-gated Na^+ (Grimes *et al*., 1995; Bennett *et al*., 2004; Onganer and Djamgoz, 2005) and K^+ channels (Laniado *et al*., 2001) and Ca^{2+}-activated K^+ channels (Schwab *et al*., 1994). These different channels may participate in a variety of processes to modulate the pattern of cell migration in the same way as different channels act to produce specific patterns of firing and synaptic release in excitable cells. One would expect that MscCa plays a central role in orchestrating the other channels because of its unique ability to transduce internally and externally generated forces into both depolarization and Ca^{2+} influx.

VI. CAN EXTRINSIC MECHANICAL FORCES ACTING ON MscCa SWITCH ON CELL MIGRATION?

A key question is what causes a cell to switch from a nonmotile to a motile phenotype and vice versa? Although there are numerous studies indicating that growth factors including tumor necrosis factor-α and transforming growth factor-β can increase cell motility by promoting the EMT (Bates and Mercurio, 2003; Masszi *et al*., 2004; Montesano *et al*., 2005; Nawshad *et al*., 2005), less well studied is the potential role of extrinsic mechanical forces in turning on cell motility. However, there are at least two key observations that support such a role. In the first place, it has been demonstrated that stationary cell fragments formed from fish keratocytes and lacking a cell nucleus or a microtubular CSK can be stimulated to polarize and undergo persistent locomotion by the application of fluid shear stress or direct mechanical poking (Verkhovsky *et al*., 1989). Similarly, the application of shear stress to quiescent *Dictyostelium* can cause CSK reorganization and stimulate cell migration (Décavé *et al*., 2003; Fache *et al*., 2005). Furthermore, these latter mechanical effects were shown to be critically dependent on the presence of external Ca^{2+} (Fache *et al*., 2005). One possible explanation is that mechanical forces alter the membrane trafficking (Maroto and Hamill, 2001; Isshiki *et al*., 2002; Rizzo *et al*., 2003) and/or the MscCa-gating properties (Hamill and McBride, 1992, 1997; McBride and Hamill, 1992), which in turn alters the $[Ca^{2+}]_i$ dynamics generated by intrinsic mechanical forces and contributes to further polarization of the cell and directional migration. Several previous studies have already discussed the possible role of the changing mechanical environment in terms of

promoting tumor malignancy, including the possible role of increasing interstitial stress and fluid pressure within a growing tumor (Sarntinoranont *et al*., 2003) and the increased tumor stiffness due to perturbed vasculature and fibrosis (Paszek *et al*., 2005) of stimulating increased cell motility and escape from the encapsulated tumor. In this case, MscCa may serve as both a trigger and mediator of tumor progression to malignancy.

Note Added in Proof

Numata, T., Shimizu, T., and Okada, Y. (*Am. J. Physiol.* **292,** C460–C467, 2007) have recently reported that TRPM7 is a stretch- and swelling-activated cation channel expressed in human epithelial cells and is blocked by Gd^{3+}. These results are consistent with the notion that several classes of mechanosensitive channels may regulate different aspects of tumor cell migration (i.e., forward protrusion and rear retraction) depending upon their differential surface distribution and interaction with downstream Ca^{2+}-sensitive effectors.

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ION CHANNELS

Revisiting TRPC1 and TRPC6 mechanosensitivity

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Abstract This article addresses whether TRPC1 or TRPC6 is an essential component of a mammalian stretch-activated mechano-sensitive Ca^{2+} permeable cation channel (MscCa). We have transiently expressed TRPC1 and TRPC6 in African green monkey kidney (COS) or Chinese hamster ovary (CHO) cells and monitored the activity of the stretch-activated channels using a fast pressure clamp system. Although both TRPC1 and TRPC6 are highly expressed at the protein level, the amplitude of the mechano-sensitive current is not significantly altered by overexpression of these subunits. In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been recently proposed to form MscCa in vertebrate cells, the functional expression of these TRPC subunits in heterologous systems remains problematic.

Keywords Mechano-electrical transduction · Cardiovascular response · Cation channel · Mechanosensitive channel · Mechanoreceptor

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Introduction

MscCa, also referred to as a stretch-activated cation channel (SAC), was recognized more than 20 years ago during patch clamp studies of chick skeletal muscle, and shown to be present in most, if not all, eukaryotic cells [4, 9, 10, 22]. MscCa displays a range of permeability properties indicating a heterogeneous composition. Initially, MscCa was proposed to derive its stretch sensitivity from the cytoskeleton (CSK) [9], but MscCa activity in CSK-deficient membrane vesicles and liposomes indicates that the channel can also be gated by forces within the bilayer [19, 31]. A membrane protein solubilization/reconstitution procedure similar to that used to identify MscL, a prokaryotic mechano-sensitive channel [27], was used to identify a *Xenopus* membrane protein fraction that reconstituted MscCa activity and was abundant in a ~80 kDa protein fraction [19]. Immunological methods demonstrated TRPC1 presence in the active fraction [19]. Furthermore,

heterologous expression of hTRPC1 increased MscCa activity, while antisense hTRPC1 reduced endogenous/background oocyte channel activity [19]. Overexpression of hTRPC1 was also reported to increase MscCa activity in CHO cells [19].

However, several recent developments warrant a novel study and discussion. First, a TRPC1−/− knockout mouse has been generated that shows no apparent phenotype [8]. This work further concludes that TRPC1 is not an obligatory component of stretch-activated and store-operated ion channel complexes in vascular smooth muscle [8]. Second, mammalian cell lines can display endogenous MscCa activity similar to that associated with hTRPC1 overexpression [19], and third, a closely related TRPC family member, TRPC6, has been reported to function as MscCa [25]. Additionally, TRPA1 has also been implicated in mechanosensation of the nematode *Caenorhabditis elegans* and was proposed as a candidate mechanosensor in mammalian hearing, although knockout studies in the mouse failed to confirm this hypothesis [5, 6, 15].

In this report, we have studied the functional expression of TRPC1 and TRPC6 in both transiently transfected CHO and COS cells in comparison with the mechano-gated K_{2P} channel TREK-1 [13]. We questioned whether the homo-multimeric TRPC1 or TRPC6 channels could be directly activated by membrane stretch. Criteria to establish direct mechanical activation of ion channels have recently been reviewed [4, 10]. The latency for current activation is expected to be less than 5 ms. Moreover, the kinetics of channel activation and deactivation should depend on the amplitude of the stimulus. Finally, the opening of an ion channel by mechanical stimulation involves the movement of a gating particle in response to force.

Our study demonstrates that the functional expression of both TRPC1 or TRPC6 is problematic, thus leaving open the question for a specific role of these subunits in MscCa activity.

Materials and methods

Cell culture, plasmid construction, transfection with DEAE-dextran or Fugene, and the electrophysiological procedures have been extensively detailed elsewhere [19, 21]. TREK-1 (accession no.: AY736359), hTRPC1 (alternatively spliced sequence accession no.: NM003304), and hTPRPC6 (accession no.: NM 004621) were transiently transfected in CHO or COS-7 cells. The same pIRES2 EGFP expression vector was used for functional expression of both channel types in transiently transfected COS and CHO cells. We routinely used 0.5 μg DNA per 35-mm-diameter plate containing ~30,000 cells. Patch pipettes were ~1.5 MΩ. Membrane stretch (ALA HSPC-1 pressure clamp) was applied as

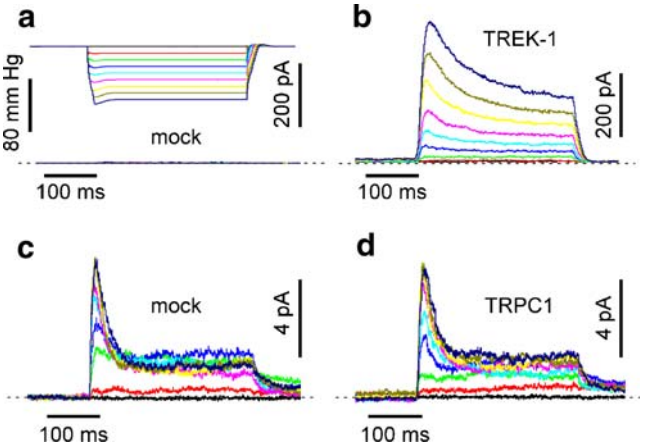

Fig. 1 Stretch-activated currents averaged across many patches in the cell-attached patch configuration in transiently transfected COS-7 cells. **a** Empty expression vector (n=16). **b** TREK-1 (n=10). **c** Mock transfection with the empty expression vector (n=70). **d** hTRPC1 (n=70). The pressure pulse protocol is shown in top panel **a**. Each *color* indicates a pressure value. The holding potential was −100 mV for panels **a** and **b** and 0 mV for panels **c** and **d**. Currents are inward in **a** and **b** and outward in **d**

previously described [14]. Routinely, the pipette solution contained (in mM): 150 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, and 10 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), pH 7.4, with NaOH, and the bath solution contained (in mM): 155 KCl, 5 EGTA, 3 Mg^{2+}, 10 HEPES, at pH 7.2. Amino-terminal EYFP-tagged mTREK-1 channel and carboxy terminal EYFP-tagged hTRPC1 were used for channel localization in transfected mammalian cells. The EYFP-mTREK-1 fusion protein showed no functional difference with the wild-type channel. In some experiments an amino terminal flag tagged hTRPC1 construct and an amino terminal HA tagged hTRPC6 were used. After mounting, specimens were observed using an epifluorescence microscope (Axioplan 2, Carl Zeiss, Oberkochen, Germany) with appropriate filters. Images were recorded with a cooled CCD camera (Coolsnap HQ, Photometrics, Tucson, AZ, USA) driven by Metavue software. Three-dimensional reconstructions and stereo pairs were made using a ZEISS confocal microscope and relevant software. *Xenopus* oocytes were injected with mRNA transcripts of EGFP-hTRPC1 and cells were studied 3–4 days after injection.

Results

Expression of TRPC1 and TREK-1 in transiently transfected COS cells

Although our earlier results presented by Maroto et al. seemed promising [19], the reported ~10-fold increase in

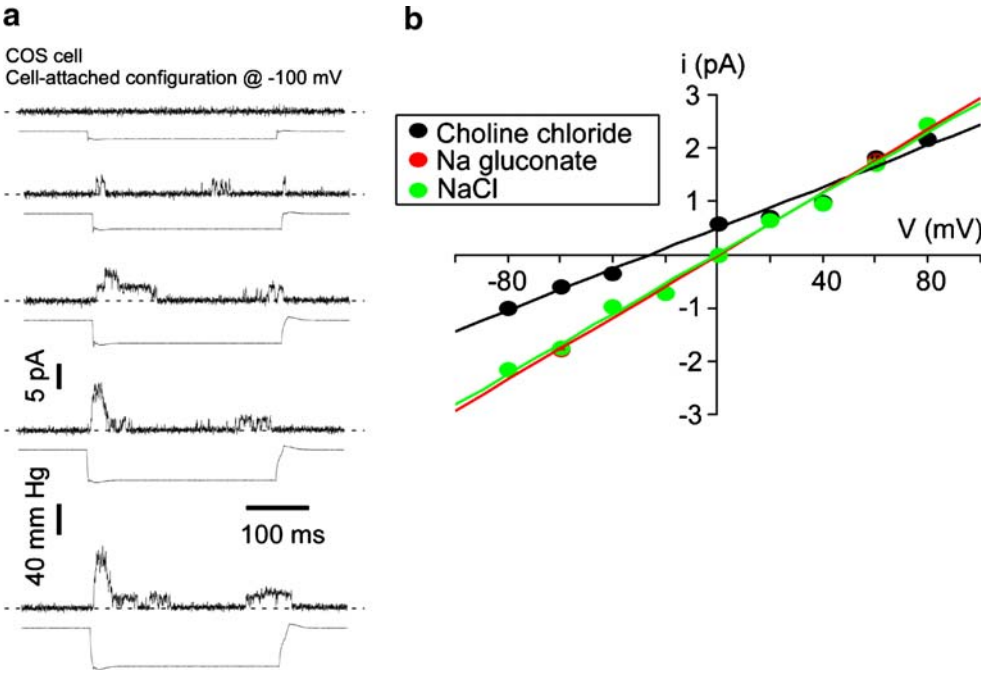

Fig. 2 **a** Cell-attached patch single channel recordings at different pressures as indicated at a holding potential of −100 mV in a mock-transfected COS-7 cells. **b** Single channel I–V curves in the presence of external 150 mM NaCl (n=4), external choline chloride (n=3), and external Na gluconate (n=3), demonstrating that channels are nonselective and cationic. Currents were recorded in mock-transfected COS cells during a pressure pulse of −30 mm Hg

MscCa activity seen with expression of hTRPC1 was much less than the 1,000-fold increase achieved with overexpression of other channels such as the mechano-sensitive K_{2P} channel TREK-1 (Fig. 1a,b). Furthermore, data presented here, and representing a much larger sample than originally studied, indicate that control COS-7 can express levels of background MscCa activity that are as high as that reported in hTRPCl-transfected cells in CHO cells [19] (Fig. 1c–d). These channels are cationic nonselective with a conductance of 28.8±0.3 pS (n=11) (Fig. 2). The mean peak current amplitudes were 9.71±1.34 pA (n=70) and 10.23±0.99 pA (n=140) for hTRPC1 and empty pIRES2 enhanced green fluorescence protein (EGFP) expressing cells, respectively. At the 0.05 level, the means are not significantly different (one-way ANOVA). After removing the silent patches (with no channel activity at −80 mm Hg), the mean peak current amplitudes became 13.59±1.46 pA (n=50) and 14.77±1.16 pA (n=97) for hTRPC1 and empty pIRES2 EGFP expressing cells, respectively. Again, at the 0.05 level, the means are not significantly different (one-way ANOVA). Moreover, the background MscCa activity is not stable and varies from cell to cell within the same culture and from experiment to experiment (Fig. 3). The basis of this variability and whether it arises through heterogeneities in endogenous TRP channel expression remains to be determined. When investigated at different pressures over a range of 80 mm Hg, no significant difference was found between the TRPC1 and the empty expression vector expressing cells, unlike the TREK-1 expressing cells [21] (Fig. 4).

Is TRPC1 expressed at the plasma membrane?

Another issue concerns the proportion of expressed hTRPC1 that is inserted in the plasma membrane [12]. Unlike with TREK-1 (Fig. 5a) and in agreement with previous studies [12], most of the expressed hTRPC1 fails to reach the plasma membrane of mammalian cells, including COS and CHO cells, but instead accumulates in the endoplasmic reticulum (Fig. 5b,d) (supplementary movies 1 and 2). TRPC1 was distributed throughout the cell and not obviously located at the cell membrane of either CHO or COS cells (Fig. 5b,d) (supplementary movies 1 and 2). In comparison, hTRPC1 expressed in frog oocytes was apparently concentrated in the surface membrane (Fig. 5c).

Expression of TRPC1 in transiently transfected CHO cells

For CHO cells (Fig. 6), over a period of 1 year, we observed a random variation of three orders of magnitude in background stretch-activated currents (Fig. 6, black squares). CHO cells transfected with hTRPC1 had the same basic response (Fig. 6, red circles). hTRPC1 transfected and untransfected cells are indistinguishable. The unitary conductance was (34±2.6 pS), similar to that reported by Maroto et al., and the reversal potential was around 0 mV [19] (not shown). The data of Maroto et al., obtained in CHO cells, are indicated in Fig. 6 as blue asterisks and they fall in the same range of current amplitude as those in nontransfected control cells observed

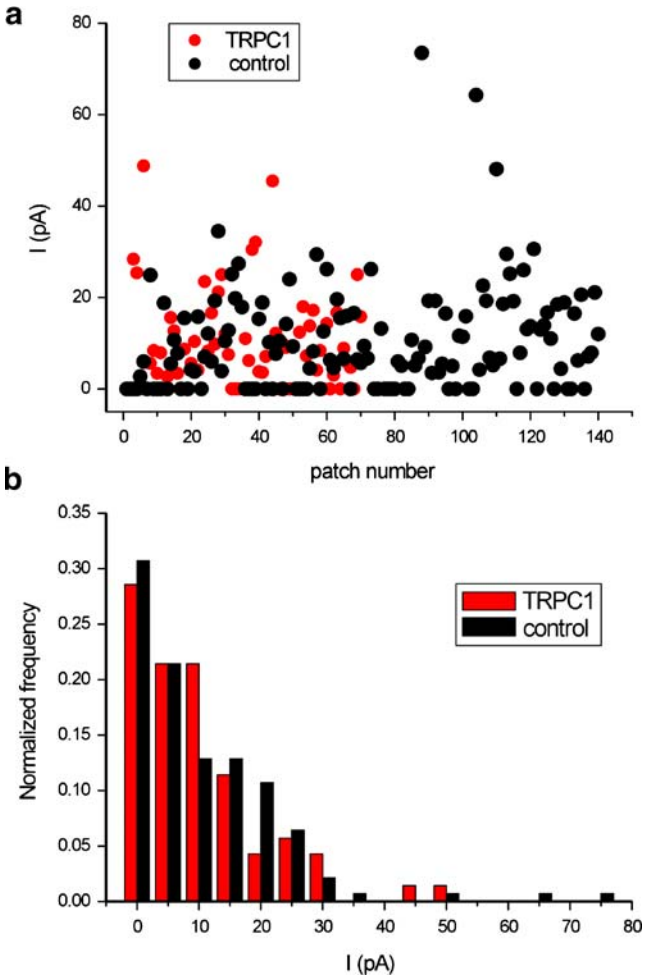

Fig. 3 **a** Peak current amplitude elicited by a −80-mm Hg pressure in the cell-attached configuration measured at −100 mV. hTRPC1 expressing COS-7 cells (shown in *red*; $n=70$) and mock-transfected COS-7 cells expressing the empty expression vector (pIRES2 EGFP) (shown in *black*; $n=140$) are illustrated. **b** Normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC1 expressing COS-7 cells ($n=70$) are shown by *red bars*, while control currents ($n=140$) are shown in *black*

in the present study [19]. Therefore, we cannot conclude that an increase in a stretch-activated current in these mammalian cells was due to the expression of the cloned hTRPC1 channel.

Expression of TRPC6 in transiently transfected COS cells

Membrane insertion of TRPC1 can be increased by cotransfection with other TRPCs [12], suggesting that endogenous TRPCs may combine with hTRPC1 to form heteromeric channels [12, 26, 28]. As a consequence, any variation in endogenous TRPC expression with clone, passage number, and/or culture condition could influence the level of expressed, as well as endogenous channel activity. TRPC6 is of particular interest because recent results indicate that hTRPC6 expression in CHO cells also leads to increased MscCa activity [25]. This would be consistent with the proposal that TRPC6 participates in the pressure-dependent myogenic contraction of cerebral arteries [29], and indeed, antisense oligonucleotides to TRPC6 attenuate the arterial smooth muscle depolarization and constriction caused by elevated intraluminal pressure [29]. However, an indirect mechanism involving diacylglycerol activation following phospholipase C stimulation has initially been proposed to account for the role of TRPC6 in the arterial myogenic contraction [29].

We have transiently transfected TRPC6 into COS cells and examined the amplitude of the stretch-activated current (Fig. 7). Preliminary experiments demonstrated that TRPC6 was highly expressed at the protein level upon transfection (Fig. 7a). However, the amplitude of the mechano-sensitive currents recorded in the cell-attached patch configuration was not different between the mock-transfected and the TRPC6-transfected cells (Fig. 7b–c), although intracellular

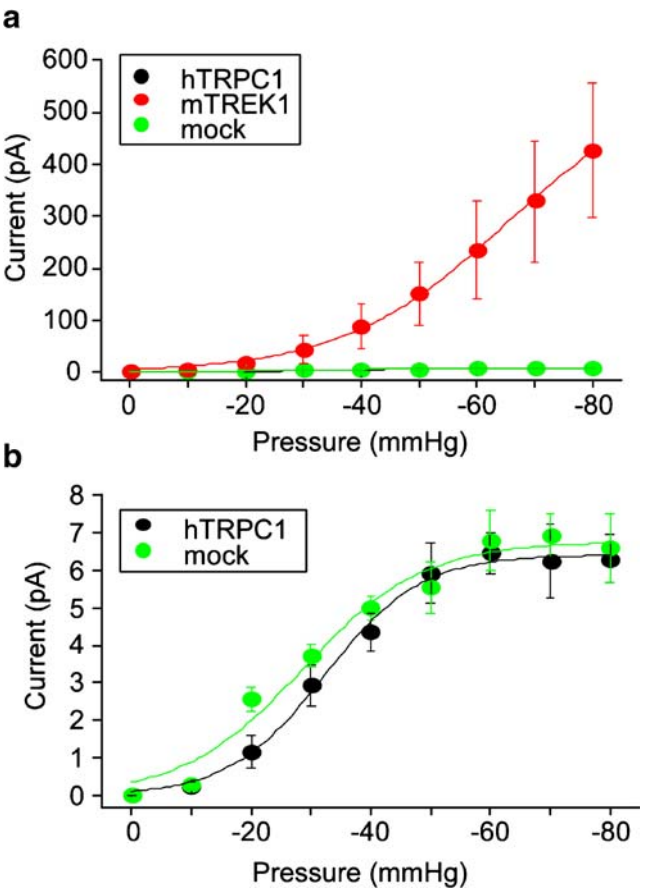

Fig. 4 **a** Pressure–response curves for COS-7 cells transfected with the empty expression vector ($n=70$), hTRPC1 ($n=70$), or mTREK-1 ($n=10$). TREK1 produces large currents, unlike TRPC1. **b** Expanded current scale for the mock and hTRPC1 conditions showing that the pressure dependence of the mock and TRPC1 cells are the same. Currents were recorded in the cell-attached patch configuration at a holding potential of −100 mV (mock and TRPC1) or 0 mV (TREK-1)

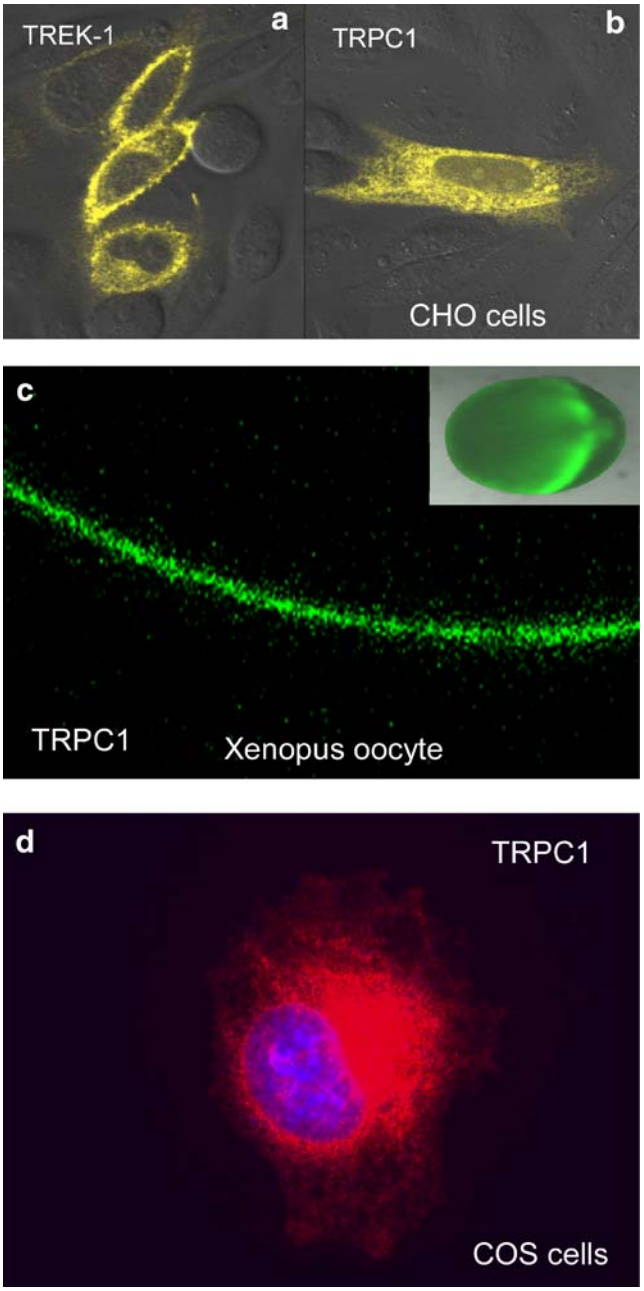

Fig. 5 **a** Confocal image of CHO cells transfected with EYFP-mTREK-1. **b** Confocal image of CHO cells transfected with hTRPC1-EYFP. Both sections are at about the same height above the coverslip. **c** A high-magnification confocal fluorescent image focused on the edge of an oocyte that had been injected 3 days earlier with an mRNA construct encoding EGFP attached to the C terminus of hTRPC1. GFP-TRPC1 is concentrated at the membrane surface of the *Xenopus* oocyte. The *insert* shows the same oocyte at lower magnification (1 mm in diameter). **d** Expression of flag-tagged hTRPC1 in transiently transfected COS cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole in blue and hTRPC1 expression is shown in *red*. Note the obvious localization of hTRPC1 in the endoplasmic reticulum

OAG application (100 μM) consistently increased channel activity, in agreement with previous reports [11]. Again, as previously observed for TRPC1, a large variability was observed within each transfection (Fig. 7d–e).

Discussion

All together these results fail to confirm a significant role for TRPC1 or TRPC6 in stretch-activated channels when expressed alone in either COS or CHO cells [19, 25]. However, in the same experiments, expression of the K_{2P} channel TREK-1 yields reproducible large-amplitude stretch-activated K^+ currents as previously described [14, 21].

Stretch-sensitivity of a channel in the patch does not prove the channel functions as a mechanotransducer either under physiological or pathological conditions. One also needs to show that modifying channel activity/expression can affect a mechanically sensitive process [3, 18]. However, TRPC6−/− mice actually show increased rather than decreased myogenic tone and are hypertensive [7]. This unexpected phenotype has been interpreted as arising from upregulation of TRPC3 that serves a similar function as TRPC6 [7]. The results of a TRPC1−/− knockout mouse are even more puzzling because this animal shows no phenotype and develops normally even though TRPC1 is the most widely expressed TRPC subunit and has been implicated to be an essential component of the store-operated channel [8]. Perhaps less surprising, because of the role of TRPC6, TRPC1 deletion does not affect vascular mechanotransduction, nor does it lead to any detectable upregulation of other TRPCs [8]. These results may indicate a normal redundancy of TRPC channels within cells in which several channels perform similar functions [2]. An analogous situation seems to apply in *Escherichia coli*, where knockout of MscL alone produces no phenotype, and only when MscS is also deleted do the cells show abnormal growth in response to osmotic stress [17]. Similar

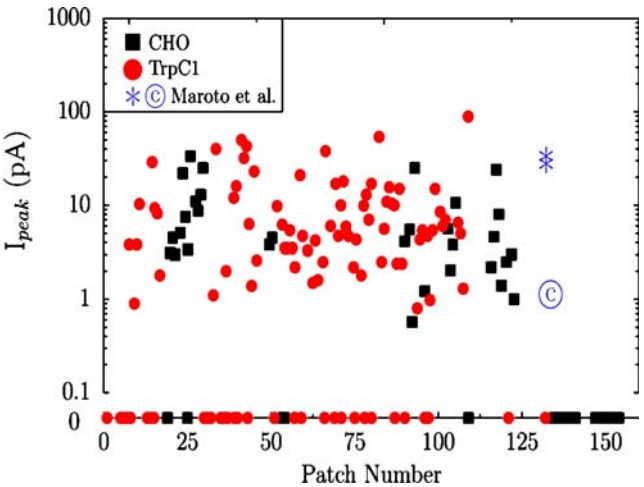

Fig. 6 Pressure-sensitive peak currents in CHO cells for patches collected over several months. Notice the log scale that suppresses the apparent scatter. Membrane potential: −90 mV. hTRPC1 transfected CHO cells (*red circles*) and control cells (*black squares*). The data of Maroto et al. [19] are shown as *blue asterisks* for TRPC1 expressing cells and the control is shown by *C*

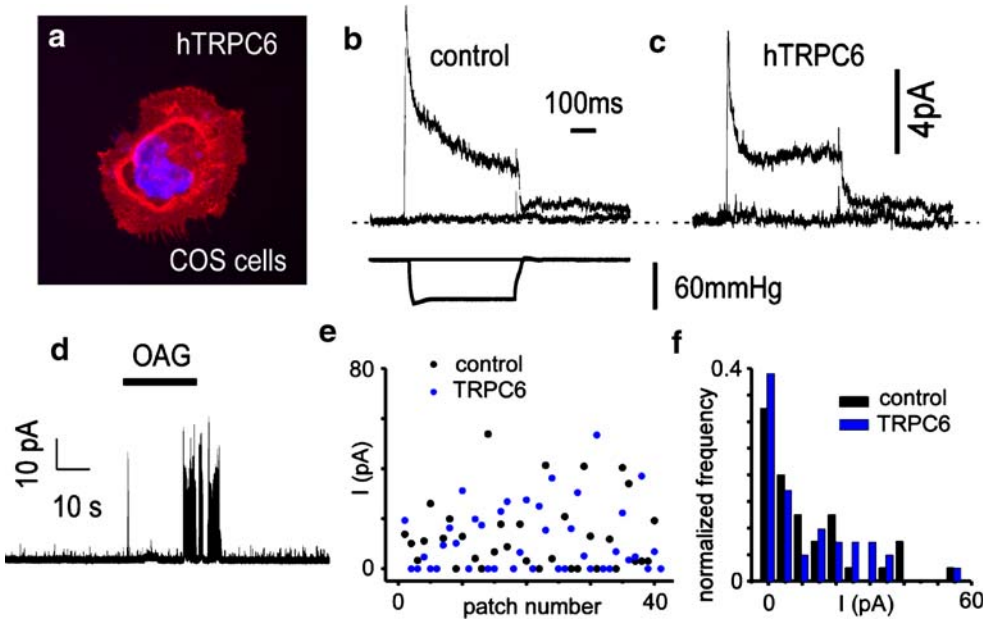

Fig. 7 **a** Expression of amino terminal HA tagged hTRPC6 in COS cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole in blue and hTRPC6 expression is shown in *red*. **b** Stretch-activated currents averaged across 40 patches in the cell-attached patch configuration in transiently transfected COS-7 cells with the empty expression vector (n=40). **c** hTRPC6 (n=41). The pressure pulse protocol is shown in panel **b**. **d** Effect of 100 μM OAG on channel activity of an inside-out patch expressing hTRPC6. **e** Pressure-sensitive peak currents in COS cells for patches collected over several transfections. hTRPC6 transfected CHO cells (*blue circles*) and control cells (*black circles*). **f** Normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC6-expressing cells (n=41) are shown by *blue bars* while control currents (n=40) are shown in *black*. In **b**–**f**, the holding potential was −100 mV and currents are inward. The pressure stimulation was −60 mm Hg

redundant mechanisms in vertebrate cells may compensate when specific MscCa activity is blocked during *Xenopus* development [30]. Clearly, the situation with MscCa contrasts with that seen with the stretch-activated K^+ channels encoded by the K_{2P} channel subunits where TREK-1 expression in either *Xenopus* oocytes or COS-7 cells results in robust mechano-sensitive K^+ currents in excised patches (≥1 nA; Fig. 1a–b) [13, 21]. Moreover, TREK-1−/− mice are more sensitive (inactivation of a stretch-activated hyperpolarizing K^+ channel) to mechanical stimuli, indicating that eukaryotic MS channels can be studied at both the molecular and organismal level [1].

The experiments of Lauritzen et al. show that expression of a channel protein can change cell structure regardless of whether or not the channel is permeant, so the proper control for transfection is far from obvious [16]. Similar changes in background currents may be elicited by cytoskeletal disrupting agents. For example, PC12 cells treated with cytochalasin-D increased the background MscCa current threefold [2.9±0.6 pS/mm Hg (n=7) vs 0.9±0.1 pS/mm Hg (n=6), data not shown]. Background MscCa are often functionally concealed, but they can be exposed by repeated stimulation [20, 23, 24]. In any case, given that all cells have background MscCa, the minimum requirement for dependable results should be a double blind experiment.

In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been proposed to form MscCa in vertebrate cells [19, 25], the functional expression of these subunits in heterologous systems remains problematic. In particular, the variable levels of background MscCa expression seen in all mammalian cell lines, and the yet-to-be-defined factors that regulate this expression complicate experiments comparing activity in mammalian sublines. Clearly, further studies are needed to identify the interacting and regulatory components required for the proper trafficking, maturation and/or functioning of this class of ion channels. This information will be critical in allowing for molecular genetic and structure-functional analysis of the vertebrate MscCa similar to that which has now commenced for the yeast stretch-activated TRPY1 channel [32].

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Chapter 7
TRPC Family of Ion Channels and Mechanotransduction

Owen P. Hamill(✉), and Rosario Maroto

Abstract Here we review recent evidence that indicates members of the canonical transient receptor potential (TRPC) channel family form mechanosensitive (MS) channels. The MS functions of TRPCs may be mechanistically related to their better known functions as store-operated (SOCs) and receptor-operated channels (ROCs). In particular, mechanical forces may be conveyed to TRPC channels through

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"conformational coupling" and/or "Ca^{2+} influx factor" mechanisms that are proposed to transmit information regarding the status of internal Ca^{2+} stores to SOCs located in the plasma membrane. Furthermore, all TRPCs are regulated by receptors coupled to phospholipases (e.g., PLC and PLA_2) that may themselves display mechanosensitivity and modulate channel activity via their generation of lipidic second messengers (e.g., diacylglycerol, lysophospholipids and arachidonic acid). Accordingly, there may be several nonexclusive mechanisms by which mechanical forces may regulate TRPC channels, including direct sensitivity to bilayer deformations (e.g., involving changes in lipid packing, bilayer thickness and/or lateral pressure profile), physical coupling to internal membranes and/or cytoskeletal proteins, and sensitivity to lipidic second messengers generated by MS enzymes. Various strategies that can be used to separate out different MS gating mechanisms and their possible role in each of the TRPCs are discussed.

7.1 Introduction

Mechanotransduction (MT) is a fundamental physiological process by which mechanical forces are transduced into electrical, ionic and/or biochemical signals. MT can span a time scale of milliseconds as in the case of a fast sensory process (e.g., in hearing and touch) to days and even years as in the case of the growth and reorganization of tissues (e.g., skin, muscle and the endothelia) in response to mechanical loading or mechanical stress. Because the plasma membrane forms the interface with the external physical world, it is continually subject to mechanical deformations arising from tissue stretch, compression, gravity, interstitial fluid pressure, fluid shear stress, and also from cytoskeleton (CSK)-generated contractile and tractile forces (Howard et al. 1988; Sachs 1988; Hamill and Martinac 2001; Perbal and Driss-Ecole 2003; Wang and Thampatty 2006; Pickard 2007). Furthermore, the membrane bilayer may be subject to local mechanical distortions caused by the insertion of lipidic second messenger molecules [e.g., diacylglycerol (DAG), arachidonic acid (AA) and lysophospholipids (LPLs)] that act by altering local packing thickness and/or lateral pressure profile, and thereby influence membrane protein conformations with consequences similar to those of global membrane deformations (Martinac et al. 1990; Hamill and Martinac 2001; Perozo et al. 2002; Kung 2005; Martinac 2007; Markin and Sachs 2007; Powl and Lee 2007). It is therefore not surprising to find that a wide range of integral and membrane-associated proteins are specialized to sense and transduce membrane distortions into different homeostatic responses. Here we focus on the seven members of the mammalian canonical transient receptor potential (TRPC1–7) channel family that provide an illustration of how very closely related membrane proteins have evolved different mechanisms for sampling their global and local mechanical environment.

7.2 Distinguishing Direct from Indirect MS Mechanisms

Because TRPCs are gated by a variety of stimuli including direct lipid bilayer stretch as well as by lipidic second messengers that are generated by membrane-associated enzymes that may themselves be mechanosensitive (MS), it is important to establish criteria that may be used to distinguish direct from indirect MS mechanisms of TRPC channel activation. Below we list some tests that may be useful in making this discrimination.

7.2.1 Stretch Activation of Channels in the Patch

The most convenient way of demonstrating an MS channel is to use a patch clamp to apply pressure or suction after formation of the giga-seal while simultaneously measuring single channel current activity (Hamill et al. 1981; Guharay and Sachs 1984). The cell-free membrane patch configurations (inside-out and outside-out) can also be used to determine if MS channel activity is retained when the cytoplasmic membrane face is perfused with solutions deficient in soluble second messengers (e.g., Ca^{2+}, cAMP, ATP). However, MS enzymes that generate membrane delimited second messengers may retain their activity following patch excision. Similarly, critical elements of the CSK involved in gating MS channels may be preserved in cell-free membrane patches (Ruknudin et al. 1991). Alternative approaches for testing CSK involvement may involve testing for MS channel activity in membrane patches formed on CSK-deficient membrane blebs induced by ATP depletion or by high ionic strength solution (Zhang et al. 2000; Honoré et al. 2006), and determining how agents that disrupt the CSK elements (e.g., cytochalasins and colchicine) affect the activity of MS channels (Guharay and Sachs 1984; Small and Morris 1994; Honoré et al. 2006).

7.2.2 Osmotic Swelling and Cell Inflation

Osmotic stress can also be used to test if a channel is MS either by swelling the cell while recording from a cell-attached patch or from the whole cell (Hamill 1983; Christensen 1987; Sackin 1989; Cemerikic and Sackin 1993; Vanoye and Reuss 1999; Spassova et al. 2006; Numata et al. 2007). The advantage of this approach is that the action of inhibitors and activators can be consistently recognized when assessed on whole cell vs patch currents. However, osmotic cell swelling also activates a number of membrane-associated enzymes, including Src kinase and phospholipase A2 (PLA_2) (Lehtonen and Kinnunen 1995; Cohen 2005a). Furthermore, although some stretch-sensitive channels are sensitive to

osmotic cell swelling and direct cell inflation, others are not (Levina et al. 1999; Vanoye and Reuss 1999; Zhang and Hamill 2000a, 2000b). One basis for this difference is that some cells may possess large excess membrane reserves in the form of folds microvilli and caveola (e.g., *Xenopus* oocytes and skeletal muscle) that can buffer rapid increases in bilayer tension (Zhang and Hamill 2000a, 2000b; Hamill 2006). Conversely, not all channels activated by cell swelling are activated by membrane stretch when applied to the patch (Ackerman et al. 1994; Strotmann et al. 2000).

7.2.3 Gating Kinetics

Another criterion that may be useful in distinguishing direct from indirect mechanisms is the delay time in activation in response to pressures steps applied to the patch with a fast pressure-clamp (McBride and Hamill 1993). Channels that are directly MS should be limited only by the conformational transitions of the channel protein, and may as a consequence show only brief delays (i.e., in the sub-ms or ms range) in their activation and deactivation (McBride and Hamill 1992, 1993). In comparison, channels dependent on enzymatic reactions and/or diffusion of second messenger may be expected to show much longer delays in opening and closing (e.g., ≥1 s). These kinetic measurements are best made in the cell-attached or cell-free patch using the pressure clamp to apply pressure steps (1–5 ms rise time) in order to measure the latency in activation and deactivation of the channels (McBride and Hamill 1992, 1993, 1995, 1999; Besch et al. 2002). Rapid activation and deactivation kinetics have been reported for the mechanosensitive Ca^{2+}-permeable cation channel (MscCa) that is formed by TRPCs in *Xenopus* oocytes (Hamill and McBride 1992; McBride and Hamill 1992, 1993) and the expressed TRAAK channel [i.e., a TWIK (tandem of pore domains in a weak inward rectifier K^+ channel)-Related Arachidonic Acid stimulated K^+ channel; Honoré et al. 2006]. In contrast, both the MscK expressed in snail neurons, which may also be a two-pore-domain K^+-channel-like TRAAK (Vandorpe and Morris 1992), and the cation channel formed by TRPC6 show long activation delays of 5–10 s (Small and Morris 1994; Spassova et al. 2006). However, because the delays can be abolished by mechanical and/or chemical CSK disruption it seems more likely that the delays reflect CSK constraint of the bilayer, which prevents rapid transmission of tension to the channel (Small and Morris 1994; Hamill and McBride 1997; Spassova et al. 2006). No studies measuring possible delays in pressure activation of TRPs suspected of being indirectly MS have been performed to date. In the case of the activation of TRPV4, which has been functionally linked to MS PLA_2 generation of AA and its subsequent metabolism to 5′, 6′-epoxyeicosatrienoic acid (5′6′-EET) by cytochrome P450 epoxygenase activity (Vriens et al. 2004; Watanabe et al. 2003), whether the apparent lack of stretch sensitivity

was overlooked because of long delays and slow channel activation still needs to be determined (Strotmann et al. 2000).

7.2.4 The Use of MS Enzyme Inhibitors

A further strategy for implicating potential MS enzymatic steps in channel activation is to test specific enzyme inhibitor on channel activity. For example, bromoenol lactone (BEL) can be used as a selective blocker of the Ca^{2+}-independent phospholipase A2 ($iPLA_2$), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4,d] pyrimidine) is a Src tyrosine kinase blocker, and U73122 can be used to block phospholipase C (PLC) (reviewed by Hamill and Maroto 2007). Using this approach, it has been reported that the stretch sensitivity of a 30 pS cation channel measured in cell-attached patches formed on arterial smooth muscle is abolished by perfusion of the whole muscle cell with U73122, indicating that the channel may derive its stretch sensitivity from a MS $iPLA_2$ (Park et al. 2003). However, a different study reported that U73122 was ineffective in blocking the stretch sensitivity of a similar 30 pS cation channel measured in inside-out patches isolated from CHO cells transfected with hTRPC6 (Spassova et al. 2006). In another example that involves the excess Ca^{2+}-influx that occurs in dystrophic muscle, and has been proposed to be mediated by TRPC-dependent MS and/or store-operated channels (SOC) (Vandebrouck et al. 2002; Ducret et al. 2006), it was shown that the Ca^{2+} influx could be abolished by BEL (Boittin et al. 2006) and potentiated by the bee venom melittin, a potent activator of PLA_2 (Lindahl et al. 1995; Boittin et al. 2006).

7.2.5 Reconstitution of MS Channel Activity in Liposomes

The most unequivocal method for distinguishing direct from indirect mechanosensitivity is to examine whether the detergent-solubilized channel protein retains stretch sensitivity when reconstituted in pure liposomes. So far this criterion has been applied to several MS channels in prokaryotes and MscCa expressed in the frog oocyte (Sukharev et al. 1993, 1994; Sukharev 2002; Kloda and Martinac 2001a, 2001b; Maroto et al. 2005). This approach also offers the potential of definitive evidence on whether lipidic second messengers (e.g., DAG, AA, LPLs and 5′6′-EET) activate the channel by binding directly to the channel protein and/or the surrounding lipid without the requirement of additional proteins and/or enzymatic steps. Furthermore, the same approach may be used to determine whether multi-protein component MS signaling complexes can be functionally reconstituted (e.g., TRPV4/PLA_2/P450 and TRPCs/PLC).

7.3 Extrinsic Regulation of Stretch Sensitivity

It seems highly unlikely that the stretch sensitivity of different membrane channels will be accounted for by a single structural domain analogous to the S-4 voltage sensor domain common to voltage-gated Na^+, K^+ and Ca^{2+} channels (Hille 2001). This is because even the relatively simple peptide channels gramicidin and alamethicin, which possess dramatically different structures and gating mechanisms, exhibit stretch sensitivity (Opsahl and Webb 1994; Hamill and Martinac 2001; Martinac and Hamill 2002). Furthermore, stretch sensitivity is not a fixed channel property, but rather can undergo significant changes with changing extrinsic conditions. For example, mechanical and/or chemical disruption of the CSK can either enhance or abolish the stretch sensitivity of specific channels (Guharay and Sachs 1984; Hamill and McBride 1992, 1997; Small and Morris 1994; Patel and Honoré 2001; Hamill 2006); changes in bilayer thickness (Martinac and Hamill 2002), membrane voltage (Gu et al. 2001; Morris and Juranka 2007), or dystrophin expression (Franco-Obregon and Lansman 2002) can switch specific MS channels between being stretch-activated to being stretch-inactivated; specific lipids (Patel and Honoré 2001; Chemin et al. 2005), nucleotides (Barsanti et al. 2006a, and references therein) and increased internal acidosis (Honoré et al. 2002; Barsanti et al. 2006b) can convert MS channels into constitutively open 'leak' channels. The basis for many of these changes involves changes in the way the bilayer, CSK and/or extracellular matrix conveys mechanical forces to the channel protein. The practical consequence of this plasticity is that the specific conditions associated with reconstitution and/or heterologous expression may alter the stretch sensitivity of the channel.

7.4 Stretch Sensitivity and Functional MT

Although stretch sensitivity measured in the patch can be used to demonstrate a channel protein is MS at the biophysical level, it cannot prove that the channel functions as a physiological mechanotransducer because conditions associated with the giga-seal formation can increase the stretch sensitivity of the membrane patch (Morris and Horn 1991; Zhang and Hamill 2000b; Hamill 2006). Indeed, many structurally diverse voltage- and receptor-gated channels (e.g., Shaker, L-type Ca^{2+} channels, NMDA-R, S-type K^+ channels), as well as the simple model peptide channels alamethicin and gramicidin A, display stretch sensitivity in patch recordings (Opsahl and Webb 1994; Paoletti and Ascher 1994; Martinac and Hamill 2002; Morris and Juranka 2007). In order to demonstrate functionality one also needs to show that blocking the channel (pharmacologically and/or genetically) inhibits a mechanically induced cellular/physiological process.

7.5 General Properties of TRPCs

The designation TRP originated with the discovery of a *Drosophila* mutant that showed a transient rather than a sustained receptor potential in response to light (Cosens and Manning 1969). This response was subsequently shown to involve a PLC-dependent Ca^{2+}-permeable cation channel (Minke et al. 1975; Montell and Rubin 1989; Minke and Cook 2002). Beginning in the mid 1990s, seven mammalian TRP homologs were identified that, together with the *Drosophila* TRP, make up the canonical TRP (TRPC) subfamily. Other TRP subfamilies include TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPM (melastatin), TRPML (mucolipid), TRPN (NOMPC) and TRPY (yeast); these combine with TRPC to form the TRP superfamily (Montell 2005; Nilius and Voets 2005; Ramsey et al. 2006; Saimi et al. 2007; Nilius et al. 2007). In addition to the TRPCs, specific members of the other TRP subfamilies have also been implicated in MT so that the MS mechanisms discussed here may also apply to these channels (Walker et al. 2000; Palmer et al. 2001; Zhou et al. 2003; Muraki et al. 2003; Nauli and Zhou 2004; O'Neil and Heller 2005; Voets et al. 2005, Saimi et al. 2007; Numata et al. 2007).

The proposed transmembrane topology of TRPCs is reminiscent of voltage-gated channels – sharing six transmembrane spanning helices (TM1–6), cytoplasmic N- and C-termini and a pore region between TM5 and TM6 – but lacking the positively charged residues in the TM4 domain that forms the voltage sensor. TRPC channels also share an invariant sequence in the C-terminal tail called a TRP box (E-W-K-F-A-R), as well as 3–4 N-terminal ankyrin repeats. Although the ankyrin repeats may act as gating springs for MS channels (Howard and Bechstedt 2004) and the positively charged residues in the TRP box may interact directly with the membrane phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rohács et al. 2005) their exact roles remain to be verified (Vazquez et al. 2004a; Owsianik et al. 2006). The TRPCs share very little sequence identity in the region that is C terminal of the TRP box, except for the common feature of calmodulin (CaM)- and inositol 1,4,5-trisphosphate receptor (IP_3R)-binding domains that have been implicated in Ca^{2+} feedback inhibition and activation by store depletion, respectively (Kiselyov et al. 1998; Vaca and Sampieri 2002; Bolotina and Csutora 2005). Based on sequence homology, the TRPCs have been subdivided into the major subgroups of TRPC1/4/5 (showing 65% homology) and TRPC3/6/7 (showing 70–80% homology). TRPC2 is grouped alone because it forms a functional channel in rodents but not in humans (i.e., it is a pseudogene in humans because of the presence of multiple stop codons within its open reading frame).

7.5.1 TRPC Expression

TRPCs are widely expressed in mammalian tissues with some cell types expressing all seven members and others expressing only one or two (Riccio et al. 2002b; Goel et al. 2006; Antoniotti et al. 2006; Hill et al. 2006). Cells that express only one

TRPC may prove particularly useful models for dissecting out specific TRPC functions. However, to justify this role it is necessary to verify that their selective expression is reflected at both the transcriptional and protein levels. This is important because low turnover proteins may require little mRNA, and high mRNA levels need not translate into high membrane protein levels (Andersen and Seilhamer 1997). Another caveat is that TRPC expression patterns can vary significantly during development (see Strübing et al. 2003), and with culture conditions (e.g., presence or absence of growth factors). For example, TRPC1 expression is upregulated by (1) serum deprivation, which leads to increased proliferation of pulmonary arterial smooth muscle cells (Golovina et al. 2001), (2) tumor necrosis factor α, which enhances endothelial cell death (Paria et al. 2003), and (3) vascular injury in vivo, which contributes to human neoitimal hyperplasia (Kumar et al. 2006). Also, TRPC6 expression in pulmonary arterial smooth muscle cells is enhanced in idiopathic pulmonary hypertension and by platelet-derived growth factor (Yu et al. 2003, 2004). Compared with mammalian cells there is less information on TRPC expression in lower vertebrates. For example, although a TRPC1 homologue has been identified in *Xenopus* oocytes, a systematic study of expression of other TRPs in lower vertebrates has not yet been carried out (Bobanović et al. 1999).

7.5.2 TRPC Activation and Function: Mechanisms of SOC and ROC

Studies of TRPC activation and function are complicated by their polymodal activation and splice variants that display different activation mechanisms (see Ramsey et al. 2006). However, all TRPCs are regulated by PLC-coupled receptors (i.e., G-protein-coupled receptors or tyrosine kinase receptors). PLC hydrolyzes a component of the bilayer, PIP_2, into two distinct messengers – the soluble inositol 1,4,5-trisphosphate ($InsP_3$) that activates the IP_3R in the endoplasmic reticulum (ER) to release Ca^{2+} from internal stores – and the lipophilic DAG, which may regulate TRPs indirectly via protein kinase C (PKC) or by interacting directly with TRPCs in a membrane delimited manner (Hofmann et al. 1999; Delmas et al. 2002; Clapham 2003; Ahmmed et al. 2004; Ramsey et al. 2006). Although all TRPCs could be classified as receptor-operated channels (ROCs, but see Janssen and Kwan 2007), they are more often subdivided into either (1) SOCs, based on their sensitivity to Ca^{2+} store depletion, or (2) ROCs based on both their activation by DAG, AA or their byproducts, and their insensitivity to Ca^{2+} store depletion (Hofmann et al. 1999; Shuttleworth et al. 2004). To be classified as a SOC, the channel should be gated by a variety of procedures that share only the common feature of reducing Ca^{2+} stores, which may or may not depend on IP3R signaling (see Parekh and Putney 2005). Unfortunately, there have been conflicting reports for all seven TRPCs on whether they function as SOCs, ROCs or both. Furthermore, the nature of the mechanism(s) that activates SOCs remains controversial, with at least two main classes of mechanism in contention. One mechanism depends upon a soluble

Ca^{2+} influx factor (CIF) that is released from depleted Ca^{2+} stores and diffuses to the plasma membrane where it activates the SOC, possibly by releasing inhibitory CaM from $iPLA_2$, which generates LPLs and AA (see Bolotina and Csutora 2005) Direct support for this CIF-CaM-$iPLA_2$-LPL model has come from the demonstration that functional $iPLA_2$ is required for SOC activation, displacement of CaM from $iPLA_2$ activates SOC, and the direct application of LPLs (but not AA) to inside-out patches activates SOCs (Smani et al. 2003, 2004). On the other hand, the generality of this model has been questioned by the finding that BEL, an $iPLA_2$ inhibitor, does not block thapsigargin-induced Ca^{2+} entry in RBL 2H3 or bone-marrow-derived mast cells (Fensome-Green et al. 2007).

The second main SOC mechanism involves conformational coupling (i.e., "CC" mechanism) between the SOC and a molecule located in the ER that transmits information regarding [Ca^{2+}] levels in internal stores. This mechanism has received its strongest support with the discovery of STIM (stromal interaction molecule), a resident ER protein with a putative Ca^{2+} binding domain in the ER lumen. Following Ca^{2+} store depletion, STIM has been shown to undergo rapid clustering and increased interactions with elements of the plasma membrane (Liou et al. 2005; Roos et al. 2005). Furthermore, it has been demonstrated that the STIM1 carboxyl-terminus activates native SOC, Ca^{2+} release-activated currents (I_{CRAC}) and TRPC1 channels (Huang et al. 2006). It may turn out that both CIF and CC mechanisms can operate in a redundant manner to activate SOCs, with the CIF mechanism conferring indirect MS on SOC via a MS $iPLA_2$ (Lehtonen and Kinnunen 1995), and the CC mechanism allowing for direct transmission of mechanical force via a direct STIM–SOC physical connection.

7.5.3 TRPC–TRPC Interactions

Assuming that a cell expresses all seven TRPC subunits, and that four TRPC subunits are required to form a channel (i.e., as a homotetramer or heterotetramer), then there could be as many as 100 different TRPC channels with different neighbor subunit interactions. However, this number would be much lower if only certain TRPC–TRPC combinations are permitted. Two different models have been proposed to underlie the permissible TRPC interactions: the homotypic model, which allows subunits interactions only within each major subgroup – with TRPC1/4/5 combinations forming SOCs and TRPC3/6/7 combinations forming ROCs – (Hofmann et al. 2002; Sinkins et al. 2004); and the heterotypic model, which permits interactions both within and between members of the two subgroups. In a specific heterotypic model developed by Villreal and colleagues it is proposed that TRPC1, 3 and 7 combine to form SOCs (i.e., without participation of TRPC4 and TRPC6) while TRPC3, 4, 6 and 7 combine to form ROCs (i.e., without TRPC1 participation) (Zagranichnaya et al. 2005; Villereal 2006). In this case, the TRPC1 role is limited to forming a SOC and TRPC4 and TRPC6 are limited to forming ROCs. However, in contradiction of an exclusive ROC role for TRPC4, it has been

reported that SOC currents in adrenal cells are abolished by TRPC4 anti-sense treatment (Phillip et al. 2000) and that endothelial cells isolated from TRPC4 knockout mice lack SOC activity (Freichel et al. 2003, 2004). In contrast to the exclusive roles of TRPC1, 4 and 6, TRPC3 and TRPC7 can participate in forming both SOCs and ROCs (Zagranichnaya et al. 2005). The validity of the different models has yet to be resolved. However, whereas the homotypic model has been based mostly on gain-of-function results from TRPC overexpression studies, the heterotypic model has been based mostly on loss-of-function results from TRPC suppression studies. At least one complication with the overexpression studies is related to the finding that different levels of specific TRPC expression can influence the function displayed in the transfected cell. In particular, low TRPC3 expression results in increased SOC activity, while high TRPC3 expression results in increased ROC activity (Vazquez et al. 2003). This variation may occur because high expression levels favor TRPC3 homotetrameric channels, whereas low TRPC3 expression allows for heterotetrameric channels with incorporation of endogenous subunits as well as exogenous TRPC (Brereton et al. 2001; Vazquez et al. 2003). Differences in channel function may also arise depending upon whether the cell is permanently or transiently transfected, presumably because stable transfection provides added time for adaptive changes in endogenous protein expression (Lièvremont et al. 2004).

7.5.4 TRPC Interactions with Scaffolding Proteins

TRPCs also interact with a variety of regulatory and scaffolding proteins that may add further diversity and segregation of the channels (Ambudkar 2006). In particular, it has been shown that several TRPCs assembly into multi-protein and lipid signaling complexes that result in physical and functional interactions between the plasma membrane, and CSK and ER resident proteins. These interactions may also allow for mechanical forces to be conveyed via a tethered mechanism to gate the channel (Guharay and Sachs 1984; Howard et al. 1988; Hamill and Martinac 2001; Matthews et al. 2007; Cantiello et al. 2007). Alternatively, the interactions may also serve to constrain the development or transmission of bilayer tension to the TRPC channel and thereby "protect" it from being mechanically activated (Small and Morris 1994; Hamill and McBride 1997). For all TRPCs, the C-terminal coiled-coil domains and the N-terminal ankyrin repeats have the potential to mediate protein-CSK interactions. All TRP family members also encode a conserved proline rich sequence LP(P/X)PFN in their C termini that is similar to the consensus binding site for Homer, a scaffold protein that has been shown to facilitate TRPC1 interaction with IP_3R – disruption of which has been proposed to promote SOC activity (Yuan et al. 2003). In particular, TRPC1 mutants lacking Homer protein binding sites show diminished interaction between TRPC1 and IP_3R and the TRPC1 channels are constitutively active. Moreover, co-expression of a dominant-negative form of Homer increases basal TRPC1 channel activity (Yuan et al. 2003). Another protein, I-mfa,

which inhibits helix-loop-helix transcription factors, also binds to TRPC1 and blocks SOC function (Ma et al. 2003). TRPC1 also possesses a dystrophin domain within its C-terminus (Wes et al. 1995) that may allow for interaction with dystrophin – the major CSK protein in skeletal muscle – and this could possibly explain why the absence of dystrophin in Duchenne muscular dystrophic muscle results in TRPC1 channels being abnormally gated open (see Sect. 7.6.1.4). TRPC1 also shows a putative caveolin-1-binding domain that may promote its functional recruitment into lipid rafts and increase SOC activity (Lockwich et al. 2000; Brazier et al. 2003; Ambudkar 2006). As mentioned previously, TRPC1 also interacts with STIM, the putative ER Ca^{2+} sensor molecule that regulates SOC function (Huang et al. 2006). Junctate – another IP_3R-associated protein – interacts with TRPC2, 3 and 5, but apparently not with TRPC1, to regulate their SOC/ROC function (Treves et al. 2004; Stamboulian et al. 2005). In pulmonary endothelial cells, TRPC4 is localized to cell–cell adhesions in cholesterol-rich caveolae and has been shown to interact with the spectrin CSK via the protein 4.1 (Cioffi et al. 2005; Torihashi et al. 2002). Furthermore, either deletion of the putative 4.1 protein binding site on the TRPC4 C-terminus or addition of peptides that competitively bind to that site are able to reduce SOC activity. Another site for TRPC4–CSK interaction involves the PSD-95/disc large protein/zona occludens 1 (PDZ) binding domain located at the TRPC4 distal C-terminus, which binds to the Na^+/H^+ exchange regulatory factor (NHERF) scaffolding protein (Mery et al. 2002; Tang et al. 2000). TRPC6 interacts with the stomatin-like protein podocin, which may modulate its mechano-operated channel (MOC) function in the renal slit diaphragm (Reiser et al. 2005). Interestingly, another stomatin homolog, MEC-2, was proposed to link the putative MS channel to the microtubular CSK in *Caenorhabditis elegans* neurons (Huang et al. 1995) but most recently has been implicated, along with podocin, in regulating MS channel function by forming large protein–cholesterol complexes in the plasma membrane (Huber et al. 2006).

In summary, TRPCs undergo dynamic interactions with various scaffolding proteins that may act to inhibit or promote a particular mode of channel activation. Any one of these interactions may be important in modulating MS of TRPCs by focusing mechanical force on the channel or constraining the channel and/or bilayer from responding to mechanical stretch. It may be that the right combination of TRPC proteins and accessory proteins are needed to produce channels that are not constitutively active but are responsive to factors associated with store depletion and/or mechanical stimulation.

7.5.5 TRPC Single Channel Conductance

Single channel conductance provides the best functional fingerprint of a specific channel, and is superior to identification by whole cell current properties that depend upon multiple factors including single TRPC channel conductance, gating and membrane insertion as well as functional coupling with other channel classes

(i.e., voltage- and Ca^{2+}-activated channels). For example, whole cell currents generated by expression and co-expression of TRPC1/4/5 and/or TRPC3/6/7 subgroup members show I–V relations with dramatically different rectifications (Lintschinger et al. 2000; Strübing et al. 2001). However, these differences may reflect voltage-dependent changes in any one or a combination of the above parameters. Unfortunately, compared with studies of whole cell TRPC generated currents, there have been relatively few studies of the single channel activity that is enhanced by TRPC overexpression or reduced by TRPC suppression. Furthermore, no study to date has distinguished unequivocally between channel currents arising from TRPC homomers or heteromers. To make this distinction one needs to transfect with mutant subunits that produce predictable and measurable changes in channel conductance (or channel block) depending on the subunit stoichiometry within the channel complex (see Hille 2001). Another practical issue for the comparison of different TRPC channel conductance values has been the lack of standardized recording conditions (i.e., pipette solutions with the same composition, and measured over the same voltage range). Nevertheless, a survey of the TRPC single channel values indicates roughly the following order: TRPC3 (65 pS) > TRPC5 (50 pS) > ~ TRPC4 ~ TRPC6 (~30 pS) ≥ TRPC1 (3–20 pS) for estimates made from cell-attached recordings with 100–150 mM Na^+/Cs^+, 1–4 mM Ca^{2+}/Mg^{2+} between −40 and −100 mV (Hofmann et al. 1999; Hurst et al. 1998; Kiselyov et al. 1998; Yamada et al. 2000; Vaca and Sampieri 2002; Liu et al. 2003; Bugaj et al. 2005; Maroto et al. 2005; Inoue et al. 2006; Saleh et al. 2006). The only available estimates for TRPC2 (42 pS) and TRPC7 (60 pS) were made with no divalents (Zufall et al. 2005; Perraud et al. 2001). One basis for the low conductance of TRPC1 compared with TRPC3, 4, 5, 6 and 7 is that TRPC1 lacks the negatively charged aspartate or glutamate residues at analogous positions to D633 in TRPC5 and the other TRPCs, which is situated nine residues from the end of the TM6 domain (Obukhov and Nowycky 2005). Removal of external Ca^{2+} (or Mg^{2+}) has been reported to increase TRPC1 (but not TRPC6) channel conductance and, according to some reports, cause a positive shift in TRPC1 current reversal potential (e.g., Vaca and Sampieri 2002; Maroto et al. 2005; Spassova et al. 2006). The heterogeneity in TRPC1-associated conductance measurements (i.e., 3–20 pS) may also indicate that its conductance is altered when it combines with other subunits. For example, the homomeric TRPC5 channel has a conductance of ~50 pS but the TRPC1/TRPC5 heteromer is reduced to ~10 pS (Strübing et al. 2001). In this case TRPC1 may cause structural distortion of the putative D633 ring formed by the TRPC5 monomeric assembly. The intracellular Mg^{2+} block of TRPC5 at physiological potentials that is relieved at positive potentials also appears to be mediated by D633 (Obukhov and Nowycky 2005). TRPC4 and TRPC6 may have similar voltage-dependent activities because both channels possess aspartate at positions equivalent to D633, and anionic rings at this location may space the properties of TRPC4 and TRPC6. It may also turn out that different TRPCs display multiconductance states some of which are favored by specific conditions. In any case, the conductance values listed above can serve as a baseline for future measurements of the purified/reconstituted TRPCs.

7.5.6 TRPC Pharmacology

Pharmacological tools available to study TRPCs are limited, with different agents reported to block, stimulate or have no effect on different TRPCs (Xu et al. 2005; Ramsey et al. 2006). For example, SKF-96365 blocks TRPC3- and TRPC6-mediated whole cell currents (at ~5 μM), and is considered a more selective ROC- than SOC-blocker. In contrast, 2-aminoethoxydiphenyl borate (2-APB) blocks TRPC1 (80 μM), TRPC5 (20 μM) and TRPC6 (10 μM) but not TRPC3 (75 μM), and is considered a more selective SOC- than ROC-blocker. In the case of Gd^{3+} (and La^{3+}), TRPC1 and TRPC6 are blocked but TRPC4 and TRPC5 are potentiated at 1–10 μM (Jung et al. 2003), while flufenamate blocks TRPC3, TRPC5 and TRPC7 (100 μM) but potentiates TRPC6. Amiloride, which is known to block different MscCa, has yet to be tested on TRPC channels (Lane et al. 1991, 1992; Rüsch et al. 1994). The newest anti-MscCa agent, the tarantula venom peptide GsmTX-4 (Suchyna et al. 1998, 2004; Gottlieb et al. 2004; Jacques-Fricke et al. 2006) has more recently been shown to block TRPC channels in mammalian cells but not in *Xenopus* oocytes (Hamill 2006; Spassova et al. 2006). At this stage it would be highly useful to carry out a systematic screen of the various agents reported to target MscCa and/or TRPC, including gentamicin, GsmTX-4, amiloride, 2-APB, amiloride, and SFK-96365 on ROCs as well as SOCs (Flemming et al. 2003).

7.6 Evidence of Specific TRPC Mechanosensitivity

There are several lines of evidence indicating specific TRPCs are MS, with the main evidence pointing towards TRPC1, TRPC4 and TRPC6. TRPC1 is generally considered to form a SOC that can be directly activated by LPLs, whereas TRPC4 and TRPC6 appear to form ROCs activated by AA and DAG, respectively. Here we consider whether the same mechanisms underlying SOC and ROC activity and sensitivity to lipidic second messengers is also the basis for their mechanosensitivity.

7.6.1 TRPC1

TRPC1 was the first identified vertebrate TRP homolog (Wes et al. 1995; Zhu et al. 1995); initial heterologous expression of human TRPC1 in CHO and sf9 cells showed enhanced SOC currents (Zitt et al. 1996). However, a subsequent study indicated that hTRPC1 expression in sf9 cells induced a constitutively active nonselective cation channel that was not sensitive to store depletion (Sinkins et al. 1998). This early discrepancy raises the possibility that store sensitivity (and perhaps stretch sensitivity) may depend upon a variety of conditions (e.g., expression levels, presence of

endogenous TRPCs and state of phosphorylation). For example, TRPC1 has multiple serine/threonine phosphorylation sites in the putative pore-forming region and the N- and C-termini, and at least one report indicates that PKC_{α}-dependent phosphorylation of TRPC1 can enhance Ca^{2+} entry induced by store depletion (Ahmmed et al 2004). Despite the early discrepant reports concerning TRPC1 and SOC function, many studies now point to TRPC1 forming a SOC (Liu et al. 2000, 2003; Xu and Beech 2001; Kunichika et al. 2004; for reviews see Beech 2005; Beech et al. 2003), and in cases where TRPC1 overexpression has not resulted in enhanced SOC (Sinkins et al. 1998; Lintschinger et al. 2000; Strübing et al. 2001) it has been argued that TRPC1 was not trafficked to the membrane (Hofmann et al. 2002). This does not seem to be the case for hTRPC1 when expressed in the oocyte (Brereton et al. 2000). In any case, direct involvement of TRPC1 in forming the highly Ca^{2+}-selective I_{CRAC} seems to be reduced by the recent finding that a novel protein family (i.e., CRAM1 or Orai1) forms I_{CRAC} channels (Peinelt et al. 2006; but see Mori et al. 2002; Huang et al. 2006).

7.6.1.1 A TRPC1 Homologue Expressed in Xenopus *Oocytes*

In 1999, xTRPC1 was cloned from *Xenopus* oocytes and shown to be ~90% identical in sequence to hTRPC1 (Bobanović et al. 1999). An anti-TRPC1 antibody (T1E3) targeted to an extracellular loop of the predicted protein was generated and shown to recognize an 80 kDa protein. Immunofluorescent staining indicated an irregular "punctuate" expression pattern of xTRPC1 that was uniformly evident over the animal and vegetal hemispheres. A subsequent patch clamp study also indicated that MscCa was uniformly expressed over both hemispheres (Zhang and Hamill 2000a). This uniform surface expression is in contrast to the polarized expression of the ER and phosphatidylinositol second messenger systems that are more abundant in the animal hemisphere (Callamaras et al. 1998; Jaconi et al. 1999). These results indicate that neither TRPC1 nor MscCa are tightly coupled to ER internal Ca^{2+} stores and IP_3 signaling. Originally, it was speculated that the punctuate expression of TRPC1 might reflect discrete channel clusters, but it might also indicate that these channels are localized to the microvilli that make up > 50% of the membrane surface area (Zhang et al. 2000). In another study testing the idea that xTRPC1 forms a SOC, Brereton et al. (2000) found that antisense oligonucleotides targeting different regions of the xTRP1 sequence did not inhibit IP_3-, or thapsigargin-stimulated Ca^{2+} inflow (but see Tomita et al. 1998). Furthermore, overexpression of hTRPC1 did not enhance basal or IP_3-stimulated Ca^{2+} inflow (Brereton et al. 2000). On the other hand, they did see enhancement of a LPA-stimulated Ca^{2+} influx. Interestingly, LPA also enhances a mechanically induced Ca^{2+} influx in a variety of cell types (Ohata et al. 2001). Based on this apparent lack of TRPC1-linked SOC activity, Brereton et al. (2000) proposed that TRPC1 might form the endogenous cation channel activated by the marine toxin, maitotoxin (MTX). However, in another study directly comparing the properties of the endogenous MTX-activated conductance measured in normal liver cells and

the enhanced MTX-activated conductance measured in hTRPC1-transfected liver cells, Brereton et al. (2001) found that the endogenous conductance showed a higher selectivity for Na^+ over Ca^{2+}, and a higher sensitivity to Gd^{3+} block ($K_{50\%\ block}$ = 1 μM vs 3 μM) compared with the enhanced conductance. These differences may indicate that other endogenous TRPC subunits combine with TRPC1 to form the endogenous MTX-activated conductance, whereas the enhanced MTX-activated conductance is formed exclusively by hTRPC1 homotetramers (Brereton et al. 2001). Finally, unlike in hTRPC1-transfected oocytes, hTRPC1-transfected rat liver cells did show an increased thapsigargin-induced Ca^{2+} inflow (Brereton et al. 2000, 2001).

7.6.1.2 MTX and TRPCs

Evidence from several studies indicates that oocyte MTX-activated conductance may be mediated by MscCa (Bielfeld-Ackermann et al. 1998; Weber et al. 2000; Diakov et al. 2001). In particular, both display the same cation selectivity, both are blocked by amiloride and Gd^{3+}, both are insensitive to flufenamic and niflumic acid, and both have a single channel conductance of ~25 pS (i.e., when measured in symmetrical 140 mM K^+ and 2 mM external Ca^{2+}). Because MTX is a highly amphipathic molecule (Escobar et al. 1998), it may activate MscCa by changing bilayer mechanics, as has been proposed for other amphipathic agents that activate or modulate MS channel activity (Martinac et al. 1990; Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998, Perozo et al. 2002).

7.6.1.3 TRPC1 and Volume Regulation

To directly test whether TRPC1 might be MS, Chen and Barritt (2003) selectively suppressed TRPC1 expression in rat liver cells and measured the cellular response to osmotic cell swelling. Liver cells are known to express MscCa (Bear 1990), and previous studies had shown that osmotic swelling of epithelial cells activates an MscCa-dependent Ca^{2+} influx that stimulates Ca^{2+}-activated K^+ efflux accompanied by Cl^-/H_2O efflux and regulatory volume decrease (RVD; Christensen 1987). However, contrary to expectations, hypotonic stress actually caused a greater swelling and faster RVD in the TRPC1 suppressed liver cells than in the control liver cells (Chen and Barritt 2003). This may occur because TRPC1 suppression results in a compensatory overexpression of other transport mechanisms that enhance both cell swelling and RVD. It should also be recognized that cell swelling does not always activate MscCa. For example, although hypotonic solution activates a robust Ca^{2+}-independent Cl^- conductance in *Xenopus* oocytes that should contribute to RVD, it fails to activate the endogenous MscCa (Ackerman et al. 1994; Zhang and Hamill 2000a).

7.6.1.4 TRPC1 in Muscular Dystrophy

Both TRPC1 and MscCa are expressed in skeletal muscle and both have been implicated in the muscular degeneration that occurs in Duchenne muscular dystrophy (DMD). In particular, muscle fibers from the *mdx* mouse (i.e., an animal model of DMD) show an increased vulnerability to stretch-induced membrane wounding (Yeung and Allen 2004; Allen et al. 2005) that has been linked to elevated $[Ca^{2+}]_i$ levels caused by increased Ca^{2+} leak channel activity (Fong et al. 1990) and/or abnormal MscCa activity (Franco and Lansman 1990). Based on the observation that the channel activity was increased by thapsigargin-induced store depletion, it was proposed that the channel may also be a SOC belonging to the TRPC family (Vandebrouck et al. 2002, see also Hopf et al. 1996). To test this idea, *mdx* and normal muscle were transfected with anti-sense oligonucleotides designed against the most conserved TRPC regions. The transfected muscles showed a significant reduction in expression of TRPC-1 and -4 but not -6 (all three TRPCs are expressed in normal and *mdx* muscle) and a decrease in Ca^{2+} leak channel activity. Previous studies indicate that MscCa behaves more like a Ca^{2+} leak channel in *mdx* mouse muscle patches (Franco-Obregon and Lansman 2002) and in some *Xenopus* oocyte patches (Reifarth et al. 1999). In a more recent study it has been confirmed that SOC and MscCa in *mdx* mouse muscle display the same single channel conductance and sensitivity to block by Gd^{3+}, SKF96365, 2APB and GsMTx-4 (Ducret et al. 2006). The presence of a dystrophin domain on the C-terminus of TRPC1 (Wes et al. 1995) could explain the shift in MscCa gating mode in *mdx* muscle that lacks dystrophin (Franco-Obregon and Lansman 2002, but see Suchyna and Sachs 2007). However, the findings that TRPC6 and TRPV2 form stretch-sensitive cation channels and are expressed in normal and *mdx* mouse skeletal muscle raises the possibility that several TRPs may contribute to MscCa activity in normal and DMD muscle (Kanzaki et al. 1999; Vandebrouck et al. 2002; Iwata et al. 2003; Muraki et al. 2003; Spassova et al. 2006).

7.6.1.5 TRPC1 Interaction with Polycystins

Further clues pointing to a MS role for TRPC1 relates to the demonstration that TRPC1 interacts with the putative MS channel TRPP2 when they are co-expressed in HEK-293 (Tsiokas et al. 1999; Delmas 2004). TRPP2 is a member of the TRPP family (polycystin) and has been shown to form a Ca^{2+}-permeable cation channel that is mutated in autosomal dominate polycystic kidney disease (ADPKD) (Nauli et al. 2003; Nauli and Zhou 2004; Giamarchi et al. 2006; Cantiello et al. 2007). TRPP2 was originally designated polycystin kidney disease 2 (PKD2) and shown to combine with PKD1, a membrane protein with a large extracellular N-terminal domain that seemed well suited for acting as an extracellular sensing antenna for mechanical forces. Both TRPP2 and PKD1 are localized in the primary cilium of renal epithelial cells that is considered essential for detecting laminar fluid flow (Praetorius and Spring 2005). However, the osmosensitive TRPV4 is also expressed

in renal epithelial cells and may also associate with TRPP2 (Giamarchi et al. 2006). It remains to be determined whether TRPC1 combines with TRPP2 in renal epithelial cells and whether knock-out of TRPC1 and/or TRPV4 blocks fluid flow detection.

7.6.1.6 TRPC1 in Mechanosensory Nerve Endings

If TRPC1 is a mechanosensory channel, it might be expected to be found in specialized mechanosensory nerve endings. To address this issue, Glazebrook et al. (2005) used immunocytochemical techniques to examine the distribution of TRPC1 and TRPC3–7 in the soma, axons and sensory terminals of arterial mechanoreceptors, and found that TRPC1, 3, 4 and 5 (but not TRPC6 and TRPC7) were expressed in the peripheral axons and the mechanosensory terminals. However, only TRPC1 and TRPC3 extended into the low threshold mechanosensory complex endings, with TRPC4 and TRPC5 limited mainly to the major branches of the nerve. Although these results are consistent with TRPC1 (and possibly TRPC3) involvement in baroreception, it was concluded that, because it was not present in all fine terminals, TRPC1 was more likely involved in modulation rather than direct MT. However, it is not clear that all fine endings are capable of transduction. Furthermore, other putative MS proteins (i.e., β and γ ENaC subunits) are expressed in baroreceptor nerve terminals (Drummond et al. 1998), in which case different classes of MS channels (i.e., ENaC and TRPC) may mediate MT in different mechanosensory nerves.

7.6.1.7 TRPC1 Involvement in Wound Closure and Cell Migration

For a cell to migrate there must be coordination between the mechanical forces that propel the cell forward and the mechanisms that promote retraction of the cell rear. The first study to implicate TRPC1 in cell migration was by Moore et al. (1998). They proposed that shape changes induced in endothelial cells by activation of TRPC1 were a necessary step for angiogenesis and cell migration. In another study, it was demonstrated that TRPC1 overexpression promoted, while TRPC1 suppression inhibited, intestinal cell migration as measured by wound closure assay (Rao et al. 2006). Based on the proposal that MscCa regulates fish keratocyte cell migration (Lee et al. 1999), and identification of TRPC1 as an MscCa subunit (Maroto et al. 2005), the role of TRPC1 in migration of the highly invasive/metastatic prostate tumor cell line PC-3 has been tested. TRPC1 activity was shown to be essential for PC-3 cell migration and, in particular, Gd^{3+}, GsMTx-4, anti-TRPC1 antibody and siRNA targeting of TRPC1 were shown to block PC-3 migration by inhibiting the Ca^{2+} dynamics that coordinated cell migration (R. Maroto et al., manuscript submitted). However, again TRPC1 may not be the only TRP channel involved in this function since TRPC6 and TRPM7 have recently been reported to be stretch-activated channels (Spassova et al. 2006; Numata et al. 2007). Irrespective of the

exact molecular identity of MscCa, it seems that this channel may be a more promising target for blocking tumor cell invasion and metastasis than integrins and metalloproteinases. This is because when a tumor cell switches from mesenchymal to amoeboid migration mode it appears to remain dependent upon Ca^{2+} influx via MscCa, whereas it becomes relatively independent of integrin and metalloproteinase activity (for review, see Maroto and Hamill 2007).

7.6.1.8 Reconstitution of xTRPC1 in Liposomes

Perhaps the most direct evidence for an MS role for TRPC1 comes from studies in which the proteins forming the oocyte MscCa were detergent-solubilized, fractionated by FPLC, reconstituted in liposomes and assayed for MscCa activity using patch recording (Maroto et al. (2005). A specific protein fraction that ran with a conductivity of 16 mS cm^{-1} was shown to reconstitute the highest MscCa activity, and silver-stained gels indicated a highly abundant 80 kDa protein. Based on previous studies that identified xTRPC1 and hTRPC1 as forming an ~80 kDa protein when expressed in oocytes (Bobanović et al. 1999; Brereton et al. 2000), immunological methods were used to demonstrate that TRPC1 was present in the MscCa active fraction. Furthermore, heterologous expression of hTRPC was shown to increase the MscCa activity expressed in the transfected oocyte, whereas TRPC1-antisense reduced endogenous MscCa activity (Maroto et al. 2005). Despite the almost tenfold increase in current density in the TRPC1-injected oocyte, the channel activation and deactivation kinetics in the two patches were similar, at least in some patches. On the other hand, in some cases the kinetics of the TRPC1-dependent channels show delayed activation and deactivation kinetics (Hamill and Maroto 2007). The basis for this heterogeneity in kinetics of TRPC1 channels remains unclear but may reflect local differences in the underlying CSK and/or bilayer or even the MscCa subunit composition that occurs with TRPC1 overexpression. Maroto et al. (2005) also demonstrated that hTRPC1 expression in CHO cells results in increased MscCa activity, consistent with a ~fivefold greater increase in channel density. Furthermore, the presence of endogenous MscCa activity is consistent with previous reports that indicate CHO cells express TRPC1 along with TRPC2, 3, 4, 5 and 6 (Vaca and Sampieri 2002).

7.6.2 TRPC2

So far there have been no studies addressing the possibility that TRPC2 is an MS channel. However, evidence does indicate that TRPC2 may function either as a ROC or a SOC depending upon cell type (Vannier et al. 1999; Gailly and Colson-Van Schoor 2001; Chu et al. 2004; Zufall et al. 2005). For example, because $TRPC2^{-/-}$ mice fail to display gender discrimination, the channel has

been implicated in pheromone detection in the rodent vomeronasal organ (VNO) (Liman et al. 1999; Zufall et al. 2005). Furthermore, because a DAG-activated channel in VNO neurons is down-regulated in TRPC2$^{-/-}$ mice and TRPC2 is localized in sensory microvilli that lack Ca^{2+} stores, it would seem that TRPC2 functions as a ROC rather than a SOC, at least in VNO neurons (Spehr et al 2002; Zufall et al. 2005). On the other hand, in erythroblasts, and possibly sperm, TRPC2 has been reported to be activated by store depletion. In both cell types, long splice variants of TRPC2 were detected (Yildrin et al. 2003), whereas VNO neurons express a short splice variant (Chu et al. 2002; Hofmann et al. 2000). In hemaetopoiesis, erthyropoietin is proposed to modulate Ca^{2+} influx via TRPC2 in possible combination with TRPC6 (Chu et al. 2002, 2004). In sperm, TRPC2 may participate in the acrosome reaction based on its inhibition by a TRPC2 antibody (Jungnickel et al. 2001). However, the fact that TRPC2$^{-/-}$ mice display normal fertility raises serious doubts regarding this role (Stamboulian et al. 2005).

7.6.3 TRPC3

TRPC3 co-localizes with TRPC1 in specialized mechanosensory nerve endings, indicating that these two TRPCs may combine to form an MS channel (see Sect. 7.6.1.6). Because TRPC3 is activated by the DAG analog 1-oleoyl-2-acetylglycerol (OAG) in a direct manner like TRPC6 (Hofmann et al. 1999), it would seem likely that it may also be sensitive to direct membrane stretch like TRPC6 (Spassova et al. 2006). However, TRPC3, unlike TRPC6, can also contribute to forming SOCs (Zitt et al. 1997; Hofmann et al. 1999; Kamouchi et al. 1999; Trebak et al. 2002; Vasquez et al. 2001; Liu et al. 2005; Groschner and Rosker 2005; Zagranichnaya et al. 2005; Kawasaki et al. 2006), and whether TRPC3 forms a SOC or a ROC has been shown to depend on levels of TRPC3 expression, indicating that subunit stoichiometry may determine activation mode (Vasquez et al. 2003; Putney et al. 2004). Finally, suppression of TRPC3 in cerebral arterial smooth muscle, while suppressing pyridine receptor-induced depolarization, does not appear to alter pressure increased depolarization and contraction, which therefore might be dependent on TRPC6 alone (Reading et al. 2005).

7.6.4 TRPC4

There is disagreement on whether TRPC4 functions as a SOC and/or ROC (Philipp et al. 1998; Tomita et al. 1998; McKay et al. 2000; Plant and Shaefer 2005). However, at least two studies by the Villreal group indicate that TRPC4 forms a ROC activated by AA rather than by DAG as in the case of TRPC3/6/7 and TRPC2 (Wu et al. 2002; Zagranichnaya et al. 2005). In particular, using siRNA and antisense strategies to reduce endogenous TRPC4 expression, TRPC4 was shown to be

required for AA-induced Ca^{2+} oscillations but not for SOC function. This AA activation may have implications for the mechanosensitivity of TRPC4 since AA has been show to activate/modulate a variety of MS channels by directly altering the mechanical properties of the bilayer surrounding the channel (Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998; Patel and Honoré 2001). Since AA is produced by PLA_2, which is itself MS (Lehtonen and Kinnunen 1995), TRPC4 may derive its mechanosensitivity from this enzyme in addition to possibly being directly sensitive to bilayer stretch. Studies of $TRPC4^{-/-}$ mice indicate that TRPC4 is an essential determinant of endothelial vascular tone and endothelial permeability as well neurotransmitter release from central neurons (reviewed by Freichel et al. 2004).

7.6.5 TRPC5

The human TRPC5 encodes a protein that is very similar to TRPC4 in its first ~700 amino acids but shows more variability in final C-terminal ~200 amino acids (Sossey-Alaoui et al. 1999; Zeng et al. 2004). Both TRPC5 and TRPC4 differ from other TRPCs in terms of possessing a C-terminal VTTRL motif that binds to PDZ domains of the scaffolding proteins EBP50 (NHERF1). However, co-expression and deletion experiments have shown that the VTTRL motif is not necessary for TRPC5 activation although it may mediate the EBP50 modulatory effects on TRPC5 activation kinetics (Obukhov and Nowycky 2004). TRPC5 (and 4) differ from the other TRPCs in that La^{3+} and Gd^{3+} cause potentiation at micromolar concentrations and block only at higher concentrations (Schaefer et al. 2000; Strübing et al. 2001; Jung et al. 2003). On this basis alone, TRPC5 and TRPC4 homotetramers would seem to be excluded from forming MscCa because Gd^{3+} has usually been reported to block MscCa at 1–10 μM (Yang and Sachs 1989; Hamill and McBride 1996). 2-APB blocks TRPC5 as well as the activating effect of Gd^{3+} possibly by directly occluding the Gd^{3+} activation site (Xu et al. 2005). TRPC5 (and TRPC4) also differ from TRPC3/6/7 in that they are not activated directly by DAG (Hofmann et al. 1999; Schaefer et al. 2000; Venkatachalam et al. 2003). However, TRPC5 is activated by LPLs including LPC when applied to excised membrane patches, but not by the fatty acid AA (Flemming et al. 2006; Beech 2006). This latter result would seem to contradict the idea that TRPC4 forms the AA-activated ROC, ARC, unless the two closely related TRPCs differ significantly in their AA sensitivity (Zagranichnaya et al. 2005).

The most intriguing functional evidence implicating TRPC5 as a putative MscCa comes from the demonstration that TRPC5, like MscCa, functions as negative regulator of neurite outgrowth (Calabrese et al. 1999; Greka et al. 2003; Hui et al. 2006; Jacques-Fricke et al. 2006; Pellegrino and Pelligrini 2007). In particular, MscCa blockers, including gentamicin, Gd^{3+} and GsmTX-4, potentiate neurite outgrowth (Calabrese et al. 1999; Jacques-Fricke et al. 2006) as does expression of a TRPC5 dominant-negative pore mutant. In contrast, overexpression of TRPC5

suppresses neurite outgrowth (Greka et al. 2003; Hui et al. 2006). Although it is tempting to suggest that TRPC5 may form MscCa in neurites, the stretch sensitivity of TRPC5 and its sensitivity to block by GsmTX-4 needs to be directly tested. Furthermore, because neurite outgrowth is potentiated by ruthenium red (a TRPV4 blocker) and suppressed by the specific TRPV4 agonist 4α-phorbol 12, 12-didecanoate, it has been suggested that TRPV4 forms the MscCa (Jacques-Fricke et al. 2006). Furthermore, in contrast to its proposed role in suppressing cell motility, TRPC5, possibly in combination with TRPC1, has also been implicated in mediating sphingosine 1-phosphate-stimulated smooth muscle cell migration (Xu et al. 2006).

7.6.6 TRPC6

The general consensus is that TRPC6 forms a ROC that is directly activated by DAG, and is insensitive to activation by IP_3 and Ca^{2+} store depletion (Boulay et al. 1997; Hofmann 1999; Estacion et al. 2004; Zagranichnaya et al. 2005; Zhang et al. 2006). Although TRPC6 is a member of the TRPC3/6/7 subfamily it shows distinct functional and structural properties. Functionally, while TRPC6 forms only a ROC, TRPC3 and TRPC7 appear capable of participating in forming both ROCs and SOCs (Zagranichnaya et al. 2005). Structurally, whereas TRPC6 carries two extracellular glycosylation sites, TRPC3 carries only one (Dietrich et al. 2003). Furthermore, exogenously expressed TRPC6 shows low basal activity compared with TRPC3, and elimination of the extra glycosylation site that is missing in TRPC3, transforms TRPC6 into a constitutively active TRPC-3 like channel. Conversely, engineering of an additional glycosylation site in TRPC3 markedly reduces TRPC3 basal activity. It will be interesting to determine how these manipulations alter the apparent MS functions of TRPC6 described below.

7.6.6.1 TRPC6 Role in Myogenic Tone

TRPC6 is proposed to mediate the depolarization and constriction of small arteries and arterioles in response to adrenergic stimulation (Inoue et al. 2001, 2006; Jung et al. 2002) and elevation of intravascular pressure consistent with TRPC6 forming a MOC as well as a ROC (Welsh et al. 2000, 2002). The cationic current activated by pressure in vascular smooth muscle is suppressed by antisense-DNA to TRPC6 (Welsh et al. 2000). Furthermore, because cation entry was stimulated by OAG and inhibited by PLC inhibitor (Park et al. 2003), it was proposed that TRPC6 forms a MS channel that is activated indirectly by pressure according to the pathway:

$\uparrow$Intravascular pressure $\rightarrow \uparrow$PLC $\rightarrow \uparrow$[DAG] $\rightarrow \uparrow$TRPC $\rightarrow \uparrow[Ca^{2+}] \rightarrow \uparrow$ myogenic tone.

In this scheme it is PLC rather than TRPC that is MS and, since all TRPCs are coupled to PLC-dependent receptors, this would imply that all TRPC could display some degree of mechanosensitivity. However, while there are reports that PLC can be mechanically stimulated independent of external Ca^{2+} (Rosales et al. 1997; Mitchell et al. 1997; Moore et al. 2002), there are more cases that indicate that the mechanosensitivity of PLC derives from stimulation by Ca^{2+} influx via MscCa (Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001). In this case, it becomes important to demonstrate that TRPC6 can be mechanically activated in the absence of external Ca^{2+} (e.g., using Ba^{2+}). There is other evidence to indicate that TRPC6 may be coupled to other MS enzymes. For example, TRPC6 is similar to TRPV4 in that it is activated by 20-hydroxyeicosatetraenoic acid (20-HETE), which is the dominate AA metabolite produced by cytochrome P-450 w-hydroxylase enzymes (Basora et al. 2003). TRPC6 may also be activated by Src family protein tyrosine kinase-mediated tyrosine phosphorylation (Welsh et al. 2002). Indeed, PP2 a specific inhibitor of Src PTKs, abolishes TRPC6 (and TRPC3) activation and strongly inhibits OAG-induced Ca^{2+} entry (Soboloff et al. 2005). OAG may operate solely through TRPC6 homomers, whereas vasopressin may act on OAG-insensitive TRPC heteromers (e.g., formed by TRPC1 and TRPC6). At least consistent with this last possibility is evidence of co-immunoprecipitation between TRPC1 and TRPC6 (Soboloff et al. 2005). A further complication is that DAG-dependent activation of PKC appears to stimulate the myogenic channels based on their block by the PKC inhibitor chelerythrine (Slish et al. 2002), whereas PKC activation seems to inhibit TRPC6 channels, which would seem more consistent with direct activation by DAG/OAG (Soboloff et al. 2005).

Despite the above evidence implicating TRPC6 as the "myogenic" channel, TRPC6-deficient mice show enhanced rather that reduced myotonic tone and increased rather than reduced responsiveness to constrictor agonist in small arteries. These effects result in both a higher elevated mean arterial blood pressure and a shift in the onset of the myogenic tone towards lower intravascular pressures, again opposite to what would be expected if TRPC6 were critical for myoconstriction (Dietrich et al. 2005). Furthermore, isolated smooth muscle from $TRPC6^{-/-}$ mice shows increased basal cation entry and more depolarized resting potentials, but both effects are blocked if the muscles are also transfected with siRNA targeting TRPC3. Based on this latter observation, it was suggested that constitutively active TRPC3 channels are upregulated in $TRPC6^{-/-}$ mice. However, the TRPC3 subunits are unable to functionally replace the lost TRPC6 function that involves suppression of high basal TRPC3 activity (i.e., the TRPC3/6 heteromer is a more tightly regulated ROC and/or MOC). In summary, although evidence indicates that TRPC6 may be a pressure- or stretch-sensitive channel and contribute to MOC, the TRPC6 knockout mouse indicates a phenotype that cannot be explained if TRPC6 alone forms the vasoconstrictor channel. It may also be relevant that another study could find no evidence that Gd^{3+}-sensitive MscCa contributes to myogenic tone in isolated arterioles from rat skeletal muscle (Bakker et al. 1999).

In the most direct study concerning TRPC6 mechanosensitivity, a stretch-activated channel current with a conductance of 25 pS (measured at +60 mV)

was activated in cell-attached patches formed on HEK293 cells transfected with hTRPC6 with a significant delay (~5 s) in turn on and turn off following a brief (2 s) pressure pulse (Spassova et al (2006). Although these long delays could indicate an indirect mechanism of stretch activation, possibly involving MS PLC (see Sect. 7.2.3), it was found that treatment of cells with cytochalasin D reduced the delays and increased stretch sensitivity, which is more consistent with the actin CSK acting as a mechanical constraint that acts to delay the transmission of tension to the bilayer. It was also found that either hypoosmotic cell swelling or application of OAG to TRPC6-transfected cells activated whole cell cation conductance that was not blocked by the PLC inhibitor U73122, apparently ruling out an indirect mechanism involving MS PLC as was previously implied (Park et al. 2003).

7.6.6.2 TRPC6 Role in Kidney Disease

Autosomal dominant focal segmental glomerulosclerosis (FSGS) is a kidney disease that leads to progressive renal kidney failure characterized by leakage of plasma proteins like albumin into the urine (proteinuria). Recently, mutations in TRPC6 were associated with familial FSGS and implicated in aberrant calcium signaling that leads to podocyte injury (Winn et al. 2005; Reiser et al. 2005). Furthermore, two of the mutants were demonstrated to be gain-of-function mutations that produce larger ROCs than the ROC currents measured in wild type TRPC6-expressing HEK-293 cells. Ultra-filtration of plasma by the renal glomeruli is mediated mainly by the podocyte, which is an epithelial cell that lies external to the glomerular basement membrane (GBM) and lines the outer endothelium of the capillary tuft located inside the Bowman's capsule. The podocyte covers the GBM and forms interdigitating foot processes that are connected by slit diaphragms, which are ultra-thin membrane structures that form a zipper-like structure at the center of the slit with pores smaller than albumin (Tryggvason and Wartovaara 2004; Kriz 2005). The podocyte-specific proteins nephrin and podocin are localized in the slit diaphragm, and the extracellular domains of nephrin molecules of neighboring foot processes interact to form the zipper structure. Podocin, a member of the stomatin family, is a scaffolding protein that accumulates in lipid rafts and interacts with the cytoplasmic domain of nephrin (Durvasula and Shankland 2006). Both nephrin and podocin have been shown to be mutated in different familial forms of FSGD. Furthermore, TRPC6 interacts with both nephrin and podocin, and a nephrin-deficiency in mice leads to overexpression and mislocalization of TRPC6 in podocytes as well as disruption of the slit diaphragm (Reiser et al. 2005). Mechanical forces play an important role in ultra-filtration, both in terms of the high transmural distending forces arising from the capillary perfusion pressure, as well as the intrinsic forces generated by the contractile actin network in the foot process that control, in a Ca-dependent manner, the width of the filtration slits. As a consequence, TRPC6 may act as the central signaling component mediating pressure-induced constriction of the slit.

In summary, two quite diverse physiological functions, myogenic tone and renal ultrafiltration, implicate TRPC6 as an MS channel, and recent evidence indicates that TRPC6 may be directly activated by stretch applied to the patch.

7.6.7 TRPC7

Since TRPC7 belongs to the same subfamily as TRPC6, and also forms a ROC activated by DAG/OAG, it might be expected to display the same direct stretch sensitivity to Ca^{2+} block as reported for TRPC6. Immunoprecipitation and electrophysiological experiments indicate that TRPC6 and TRPC7 can co-assemble to form channel complexes in A7r5 vascular smooth muscle cells (Maruyama et al. 2006). However, the same study also demonstrated that the co-assembly of TRPC7 (or TRPC73) with TRPC6 can change specific channel properties compared with the homomeric TRPC6 channel. For example, whereas increasing external Ca^{2+} from 0.05 to 1 mM suppresses currents in HEK cells transfected with TRPC7 (or TRPC3) alone, or with TRPC6/7 (or TRPC3/6) in combination, it fails to suppress currents in TRPC6-transfected cells. Therefore, apart from the constitutive opening seen with TRPC3 but not TRPC6 (see Sect. 7.5.3), TRPC3/6/7 subfamily members differ in their sensitivity Ca^{2+} block. Other studies indicate even more profound differences between TRPC7 and TRPC6 functions. For example, based on overexpression in HEK cells, it was concluded that mouse TRPC7 forms a ROC, whereas human TRPC7 forms a SOC (Okada et al. 1999; Riccio et al. 2002a). In this case, the initial explanation was that a proline at position 111 in mTRPC7 was replaced by leucine in the hTRPC7. However, hTRPC7 suppression/knockout experiments indicate that TRPC7 is required for both the endogenous SOC and ROC in HEK293 cells (Lièvremont et al. 2004; Zagranichnaya et al. 2005). Furthermore, when hTRPC7 (with leucine at position 111) was transiently expressed in HEK293 cells it enhanced ROC, but when it was stably expressed it enhanced both ROC and SOC (Lièvremont et al. 2004). In this case, the explanation was that stable transfection allowed for a time-dependent upregulation of other ancillary components that were required to couple TRPC7 to store depletion (Lièvremont et al. 2004). On the other hand, although hTRPC7 suppression in DT40 B-cells also reduced receptor/DAG-activated and store-operated Ca^{2+} entry, the latter effect appeared to arise because of increased Ca^{2+} stores and the greater difficulty in depleting them to activate SOC (Lièvremont et al. 2005). Indeed, when Ca^{2+} stores were more effectively depleted (i.e., with a combination of IP_3 and calcium chelator) there was no difference in SOC activation between wild type and $TRPC7^{-/-}$ cells (Lièvremont et al. 2005). Similar findings have been reported for TRPC7 suppression in human keratinocytes (Beck et al. 2006). A still further complication is that, in cells lacking the IP_3R, the OAG-activated current is absent but can be restored by transient IP_3R expression or by overexpression of TRPC7 (Vazquez et al. 2006). This was taken to indicate that the endogenous TRPC7 needs to interact with endogenous

proteins including regulatory IP_3R but when TRPC7 is overexpressed the other proteins are not required for OAG activation.

The above review of the TRPC literature indicates the importance of measuring directly the stretch sensitivity of different TRP channels under conditions in which the stoichiometry and molecular nature of the TRPCs forming the channel complex are well defined.

7.7 Conclusions

At least three basic mechanisms, referred to as "bilayer", "conformational coupling" and "enzymatic", may confer mechanosensitivity on TRPCs. The bilayer mechanism should operate if the TRPC channel, in shifting between closed and open states, undergoes a change in its membrane occupied area, thickness and/or cross-sectional shape. Any one of these changes would confer mechanosensitivity on the channel. A bilayer mechanism may also underlie the ability of lipidic second messengers (e.g., DAG/OAG, LPL, AA and 5′6′-EET) to directly activate TRPC channels by inserting in the bilayer to alter its local bilayer packing, curvature and/or the lateral pressure profile. The only unequivocal way to demonstrate that a bilayer mechanism operates is to show that stretch sensitivity is retained when the purified channel protein is reconstituted in liposomes. After this stage, one can go on to measure channel activity as a function of changing bilayer thickness (i.e., by using phospholipids with different acyl length chains) and local curvature/pressure profile (i.e., by using lysophospholipids with different shapes) (Perozo et al. 2002).

The second mechanism involves conformational coupling (CC), which has been evoked to account for TRPC sensitivity to depletion of internal Ca^{2+} stores. CC was originally used to explain excitation–contraction (E–C) coupling involving the physical coupling between L-type Ca^{2+} channels (i.e., dihydropyridine receptors, DHPR) in the plasma membrane and ryanodine receptors (RyR1) that release Ca^{2+} from the sarcoplasmic reticulum (SR) (Protasi 2002). Subsequently, a retrograde form of CC was discovered between the same two proteins that regulate the organization of the DHPR into tetrads and the magnitude of the Ca^{2+} current carried by DHPR (Wang et al. 2001; Paolini et al. 2004; Yin et al. 2005). Another form of CC was demonstrated associated with physiological stimuli that do not deplete Ca^{2+} stores yet activate Ca^{2+} entry through channels referred to as excitation-coupled Ca^{2+} entry channels to distinguish them from SOC (Cherednichenko et al. 2004). Interestingly, RyR1 is functionally coupled to both TRPC1-dependent SOC and TRPC3-dependent SR Ca^{2+} release (Sampieri et al. 2005; Lee et al. 2006).

A key issue for all forms of CC is whether the direct physical link that conveys mechanical conformational energy from one protein to another can also act as a pathway to either focus applied mechanical forces on the channel or alternatively constrain the channel from responding to mechanical forces generated within the bilayer. Another possibility is that reorganization or clustering of the resident ER

protein (i.e., STIM) that senses Ca^{2+} stores may alter channel mechanosensitivity by increasing the strength of CC (Kwan et al. 2003).

Some insights into these possibilities can be provided by the process of "membrane blebbing", which involves decoupling of the plasma membrane from the underlying CSK, and has been shown to either increase or decrease the mechanosensitivity of MS channels depending upon the channel (Hamill and McBride 1997; Hamill 2006). Since membrane blebbing would also be expected to disrupt any dynamic interactions between TRPC channels and scaffolding proteins it should alter TRPC function. In one case it has been reported that Ca^{2+} store depletion after, but not before, formation of a tight seal is effective in blocking the activation of SOC channels in frog oocyte patches (Yao et al. 1999). Presumably, this occurs because the sealing process physically decouples the channels from ER proteins that sense internal Ca^{2+} stores. Tight seal formation using strong suction can also reduce MscCa mechanosensitivity and gating kinetics, possibly by a related mechanism (Hamill and McBride 1992). On the other hand, it has been reported that I_{CRAC} is retained following cell "ballooning" (i.e., a form of reversible membrane blebbing) indicating that the coupling between the channel and the Ca^{2+} sensor STIM may be relatively resistant to decoupling (Bakowski et al. 2001). In any case, in order to directly demonstrate a role for CC in mechanosensitivity, one needs to show that stretch sensitivity can be altered in mutants in which TRPC–ancillary protein interactions are disrupted (see Sect. 7.5.4).

The third mechanism of mechanosensitivity relates to functional coupling between TRPCs and putative MS enzymes. Evidence indicates that the PLA_2 and Src kinase may be MS, and both enzymes have been implicated in conferring mechanosensitivity on TRPV4 (Xu et al. 2003; Vriens et al. 2004; Cohen 2005a, 2005b). PLA_2 and Src kinase have also been implicated in the activation of TRPC-mediated SOC and ROC activities (Hisatsune et al. 2004; Bolotina and Csutora 2005; Vazquez et al. 2004b). There is also evidence that indicates PLC may be MS (Brophy et al. 1993), with some reports indicating that the mechanosensitivity depends upon Ca^{2+} influx (Basavappa et al. 1988; Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001; Alexander et al. 2004) and others indicating independence of external Ca^{2+} and Ca^{2+} influx (Mitchell et al. 1997; Rosales et al. 1997; Moore et al. 2002). In either case, the combined evidence indicates that mechanical forces transduced by MscCa and/or by MS enzymes may modulate the gating of all TRP channels. The physiological and/or pathological effects of this MS modulation remain to be determined. The methods discussed in this chapter, including the application of pressure steps to measure the kinetics of MS enzyme–channel coupling and the use of membrane protein liposome reconstitution for identifying specific protein–lipid interactions should play an increasing role in understanding the importance of the different MS mechanisms underlying TRPC function.

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The role of stretch-activated and TRPC channels in prostate tumor cell migration

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The mechanosensitive Ca^{2+} channel (MscCa) transduces membrane stretch into Ca^{2+} influx and has been proposed to regulate cell locomotion by coordinating forward cell protrusion with Ca^{2+}-sensitive mechanisms that promoted rear cell retraction (Lee et al., 1999). Patch-clamp recordings indicate that MscCa is also expressed by the highly motile/invasive human prostate cancer cell line PC-3. Agents that block MscCa, including Gd^{3+} and GsMTx-4, also block PC cell migration as well as the sustained intracellular [Ca^{2+}] gradient (i.e., front low-rear high) that determines migration directionality (Maroto & Hamill, 2007). An antibody raised against the external pore region of TRPC1 blocks PC-3 cell migration, as does either suppression or overexpression of TRPC1. Together, these findings indicate that a specific density and surface distribution of TRPC1 is required to support PC cell migration and specific agents that target MscCa and/or TRPC1 may provide a novel approach to block tumor cell migration and metastasis.

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Title: *Controversies related to the stretch-activated mechanosensitve Ca2+ permeable cation channel*

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The mechanosensitive Ca2+ permeable cation channel (MscCa) transduces membrane stretch into Ca2+ influx and is widely expressed in eukaryotic cells. MscCa has been proposed to regulate a variety of functions including cell volume regulation, cell contraction and cell locomotion. Here we will describe recent efforts and controversies associated with attempts to identify the protein(s) forming MscCa and the mechanisms of MscCa gating. In addition we will describe the central role of MscCa in regulating prostate tumor cell migration and invasion.

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A stretch-activated Ca^{2+} channel regulates human prostate tumor cell migration[1]

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[3]The abbreviations used are: CSK, cytoskeleton; ECM, extracellular matrix; GsmTX-4 *Grammastola* spider venom peptide 4 kDa; MscCa, mechanosensitive Ca^{2+}-permeant channel; PC, prostate cancer; SAC, stretch activated channel; TRPC, canonical transient receptor potential channel.

ABSTRACT

In its early stages prostate cancer stays in the prostate and is not life-threatening, but without treatment it spreads to other parts of the body and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade it is important to identify the mechanisms that regulate tumor cell migration. Based on studies of fast moving fish keratocytes (25) it has been proposed that the stretch-activated mechanosensitive Ca^{2+}-permeable channel (MscCa) regulates cell migration by coordinating forward extension with mechanisms that lead to cell retraction. Here we report that MscCa is expressed in the highly migratory/invasive human prostate tumor cell line PC-3, and that sustained Ca^{2+} influx via MscCa activity is required to generate an intracellular [Ca^{2+}] gradient that determines directed migration. The nonmigratory human prostate tumor cell line LNCaP also expresses MscCa. However, in this cell the channel undergoes rapid (< 100 ms) inactivation with stretch precluding sustained Ca^{2+} influx, $[Ca^{2+}]_i$ gradient and directed cell migration. Our results indicate that MscCa and the physical/biochemical mechanisms that regulate channel gating are promising targets to block prostate tumor cell migration.

INTRODUCTION

Prostate cancer (PC) is a progressive disease involving transformation to unlimited cell growth, immortalization to escape the limits of senescence/apoptosis, and the ability to spread to distal sites (invasion and metastasis). In order for PC^3 to spread, tumor cells must migrate from the prostate, pass through blood vessels, penetrate into the secondary tumor site (typically bone), and migrate through its tissue to establish a metastasis (33). Cell migration is therefore necessary although not sufficient for invasion and metastasis, which also require the additional steps of barrier matrix breakdown, and tumor cell adherence, growth and angiogenesis at the secondary sites (12). Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in this cascade, identifying the most sensitive and susceptible step in tumor cell migration should provide a promising target to block PC metastasis (21).

Although the rates and patterns of cell migration vary among normal and cancer cells (9) they also share a basic cycle of steps involving (24,35,39) protrusion and adhesion of the front of the cell, a contraction of the cell body that leads to cell extension and finally rear retraction. A key question about this cycle concerns the mechanosensitive mechanisms that coordinate forward protrusion with rear retraction. Lee and colleagues (25) proposed from their studies of fast moving fish keratocytes that the stretch-activated MscCa (also referred to as SAC), could serve this function by its ability to "sense" and transduce membrane stretch into Ca^{2+} influx and thereby provide feedback between mechanisms that cause cell forward protrusion and those Ca^{2+}-dependent mechanisms (e.g., cell

contractility and adhesion disassembly) that promote rear retraction. Since the process of cell migration is conserved, we thought that MscCa activity might also be important for coordinating PC cell migration. In order to test this hypothesis we use patch-/pressure clamp techniques to determine if MscCa is expressed in the highly motile/invasive human prostate tumor cell line PC3, and whether the channel activity is required for PC3 cell migration. We also use high resolution intracellular Ca^{2+}-imaging techniques to measure changes in intracellular Ca^{2+} during PC-3 cell migration, and determine the role of MscCa in modulating these changes. Finally, we compare MscCa expression and intracellular Ca^{2+} changes between motile PC-3 cells and the nonmotile human prostate cell line LNCaP.

MATERIALS AND METHODS

Cultures. The human PC cell lines (ATCC, Manassa, VA) studied included the PC-3 (20), LNCaP (19) and DU-145. Cell cultures were grown in RPMI 1640 medium with 25 mM Hepes and glutamine, 8 % FCS, 1 mM Na pyruvate, 4.5 g/L glucose and antibiotics at 37ºC in a humidified 95% O_2-5% CO_2 atmosphere.

Patch-clamp recording. Standard cell-attached, patch-clamp recording was used to record single-channel currents. A custom-built pressure clamp was used to apply a gentle and reproducible suction protocols (< 10 mmHg for 10 s) in order to achieve the initial tight seal and then to mechanically stimulate the patch (15). A standardized sealing protocol was important in order to minimize any mechanically-induced changes in

channel properties before recording. The standard pipette solution contained, in mM: 100 KCl, 2 EGTA (KOH), 5 Hepes (KOH) at pH 7.4. To measure Ca^{2+} block and permeation 1 mM Ca^{2+} replaced the 2 mM EGTA. Gd^{3+} was added to the pipette solution without EGTA. In order to monitor MscCa activity before exposure to the agents, the pipette tip was filled (~300 μm) by capillary action with agent-free pipette solution, then backfilled with the agent-containing solution. The standard bath solution contained, in mM: 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (NaOH) at pH 7.4. Patch currents were filtered at 500 Hz with an 8-pole Bessel filter and digitized at 1 kHz on an IBM clone using pCLAMP (Axon Instruments, Union City, CA). Chemicals in general were purchased from Sigma (St Louis, MO) except for GsmTx-4 (Peptides International, Louisville, KY) and fluorescent agents (Invitrogen/Molecular Probes, Carlsbad, CA).

Videomicroscopy and Ca^{2+}-imaging. Cell migration was monitored at 37^{o}C by time-lapse videomicroscopy using Nomarski optics with an Epifluorescent microscope (Nikon). Fura-2 AM (5μM, 20 min) was loaded for calcium imaging assays. Images were captured at 340 and 380 nm, at 30 s and/or 1 min intervals. Images acquired with Metafluor (Universal Imaging Corp. Sunnyvale, CA) and a Photometrics Coolsnap HQ camera (Roper Scientific). Metamorph (Version 6.2, Universal Imaging Corp. Sunnyvale, CA) and Excel 2000 (Microsoft Excel, WA) were used for analysis. Conversion of pixels to μm was based on a calibrated slide. Wound assays were carried on sub confluent PC3 cells (90%) seeded on 35 mm dishes. Three scratches (~500 μm across) per dish were made using a sterile 200 μl pipette tip. As indicated, 3 μM GsMTx-4 was added to the

culture and replaced by fresh solution after 24 h. The same procedure was used in controls.

Confocal Immunofluorescence. A Zeiss LSM 510 META confocal system configured on an Axiovert 200M inverted microscope (63X 1.4 objective) was used to acquire the images (543nm excitation, green He/Ne laser) later processed with Metamorph. The distribution of ER in PC cells was studied by 10 min incubation with 200 nM BODIPY FL-thapsigargin (excitation 488, emission 510-600).

RESULTS

MscCa activity in migrating PC-3 cells. Figure 1a is a photomicrograph of a migrating PC-3 cell showing three distinct morphologically regions — a leading zone that includes the lamellipodium and lamellum, a thicker somatic region that encloses the nucleus, and an extended/stretched rear tether. Cell-attached recordings from these regions (arrowed) on over 120 different PC-3 cells indicate stretch-activated currents can be recorded in all regions (Fig. 1a). Figure 1b shows single stretch-activated channel currents recorded at -50 and 50 mV, and indicates the open channel undergoes more frequent brief openings and closings at negative compared with positive potentials. Figure 1c shows current-voltage relations measured under different ionic conditions, and indicates an inwardly rectifying channel that is permeable to both Na^+ and K^+, and shows reduced conductance in the presence of 1 mM Ca^{2+} (Fig. 1c; chord conductance measured at -50 mV was 55 pS (100 K^+:0 Ca^{2+}); 42 pS (100 Na^+:0 Ca^{2+}); 25 pS (100 K^+:1 Ca^{2+}) and

20 pS (100 Na^+:1 Ca^{2+}). Interestingly, the channel in human tumor cells displays similar gating and conductance properties as MscCa expressed in frog oocytes (44, 46, 49). In order to test if functionally significant Ca^{2+} flows through the channel under physiological conditions (~1 mM Ca^2) we used Ca^{2+}-activated K^+ (K_{Ca2+}) channel activity to assay for stretch-induced $[Ca^{2+}]_I$ changes. Figure 1d shows single outward channel currents that followed the stretch-activated inward currents. The conductance of the outward currents was ~20 pS, which is similar to the intermediate conductance K_{Ca2+} channel reported in PC-3 cells (31). Most importantly, the outward currents were never activated by stretch when Ca^{2+} was removed from the pipette solution. These results indicate that Ca^{2+} flow through MscCa under physiological conditions is sufficient to raise local $[Ca^{2+}]_i$ to levels that activate Ca^{2+}-sensitive mechanisms.

Anti-MscCa agents block PC cell migration. Testing different anti-MscCa agents on PC-3 cells, we found that 5 μM Gd^{3+}, a relatively nonspecific MS channel blocker (49), abolished MscCa activity (supplementary Fig.1a). A similar effect was seen with 3 μM GsMTx-4, which is a more specific MS channel blocker (43; supplementary Figs. 1b). Gd^{3+} and GsMTx-4 also blocked PC-3 cell migration as measured by time-lapse video microscopy and wound/scratch closure assays. Figure 2a shows video frames of PC-3 cells selected at 0, 30, and 60 minutes during migrating out from a cluster of 8 cells. Figure 2b shows representative PC-3 cell trajectories measured for periods of ≥ 1 h before, during, and after exposure to Gd^{3+} (5 μM) and GsMTx-4 (3 μM) (see also supplementary video 1). Histograms summarizing several experiments are shown in Figure 2c. Apart from blocking directional migration, the agents also altered PC-3 cell

morphology. For example, instead of a prominent ruffled lamellipodia, PC-3 cells in Gd^{3+} and GsMTx-4 lost their polarized morphology and took on a smooth, flattened "fried egg" appearance (see supplementary video 1). These effects on migration and cell morphology were rapidly reversed by washing out Gd^{3+} and GsMTx-4. Cell migration assayed by wound/scratch closure assays also confirmed the ability of GsmTx-4 to block PC-3 cell migration (supplementary Fig. 2a).

$[Ca^{2+}]_i$ dynamics in migrating PC cells. To address how MscCa activity might regulate cell migration, we measured the spatial and temporal $[Ca^{2+}]_i$ dynamics in the absence and presence of anti-MscCa agents. Figure 3a shows time-lapse fluorescent images of migrating PC-3 cell loaded with fura-2, and indicate that as the migrating cell becomes progressively extended it develops a $[Ca^{2+}]_i$ gradient increasing from the front to the rear of the cell (see also Supplementary Videos 2 and 3). This form of $[Ca^{2+}]_i$ gradient was a common trait seen in over 200 migrating PC-3 cells. The gradient collapsed with rear retraction, presumably as membrane stretch was relieved, and reversed when cells spontaneously reversed direction (Fig. 3b, supplementary video 4). Furthermore, the $[Ca^{2+}]_i$ gradient only developed in migrating PC-3 cells. For example, Fig. 3b shows two neighboring PC-3 cells in which only the migrating cell develops a gradient (see also supplementary video 4). A small proportion of migrating PC-3 cells (~10%) also exhibited fast $[Ca^{2+}]_i$ transients that spread rapidly ($\leq$ 2 min) throughout the cell and occurred with an average frequency of 2 $\pm$ 0.8 transients/h (range 1-4 transients/h in 15 cells). In some, but not all cases, the transients were immediately followed by retraction of the rear tether (see Fig. 3b & c, and supplementary video 4).

GsMTx-4 and Gd^{3+}, at the same concentrations that block PC-3 cell migration, also prevented the development of $[Ca^{2+}]_i$ gradients and transients (Fig. 3d and supplementary video 5, 10-20 cells tested in each condition). The dependence of these $[Ca^{2+}]_i$ changes on Ca^{2+} influx, was also indicated by block with 10 mM external BAPTA (supplementary video 6). Similar blocking effects were observed when internal $[Ca^{2+}]_i$ stores were depleted with 5 μM thapsigargin (data not shown) indicating the $[Ca^{2+}]_i$ dynamics depend upon Ca^{2+} influx and Ca^{2+} release from internal stores (1). We have also carried out preliminary studies measuring $[Ca^{2+}]_i$ changes in PC-3 cells plated on elastic substrate that could be stretched using glass micropipettes (25,30). The response of PC-3 cells to stretch was variable and dependent on the stage in the migratory cycle — PC-3 cells that were not extended (i.e., had no trailing tether) showed a stretch-induced global $[Ca^{2+}]_i$ elevation, whereas 3 out of 5 cells that were already extended lost their $[Ca^{2+}]_i$ gradient as their trailing tether retracted during the applied stretch (Maroto & Hamill, unpublished observations). The latter finding indicates that the relaxation of intrinsically generated forces can overcome extrinsically applied stretch.

Mechanisms that generate the $[Ca^{2+}]_i$ gradient in migrating PC cells. Several mechanisms may support the spatial $[Ca^{2+}]_i$ gradient that develops in migrating PC-3 cells. First, there is a higher probability of recording MscCa active patches towards the rear of the cell. Overall ~15% of patches showed no stretch channel activity (i.e., with suctions up to 100 mmHg). However, the percentage of null patches was highest on the lamella (24%: 10 out 42 patches) compared with the cell body (15%: 8 out 54) or the

trailing tether (11%: 3 out 28). This polarized distribution could generate a gradient of Ca^{2+} influx increasing from front to back as reported previously (38). Second, the distribution of fluorescently labeled-thapsigargin (BODIPY FL-thapsigargin) indicates ER Ca^{2+} stores are more concentrated in the cell body and rear than in the front of the cell (Fig. 4a); this would tend to further amplify any effects of polarized Ca^{2+} influx by increasing Ca^{2+}-induced Ca^{2+} release (CICR) and/or Ca^{2+} leak from internal Ca^{2+} stores (38). Finally, it has been proposed that apparent $[Ca^{2+}]_i$ gradients may develop in cells due to mitochondrial sequestration of fura-2 (34). However, our results using Mitotracker Red to measure mitochondria distributions indicate the mitochondria were concentrated around the cell nucleus and in the front half on the PC-3 cell, but were excluded from the trailing tether (Figs. 4b & 4c). In this case, the distribution would appear opposite to that required to explain the $[Ca^{2+}]_i$ gradient.

MscCa properties in migratory vs nonmigratory PC cell lines. We next asked whether MscCa is expressed in the nonmigratory PC cell LNCaP. These cells do not display the polarized morphology of PC-3 cells, but instead either are either stellar- or spindle-shaped (Fig. 5a. Supplementary Fig. 4). LNCaP cells also do not migrate as measured by wound/scratch closure assay (Supplementary Fig. 2b) or by time-lapse video-microscopy (data not shown). However, they do undergo other forms of motility that include pulsating motions, as well as multiple mini-lamellipodia and blebs that transiently protrude around the perimeter of the cell (Supplementary video 7). Surprisingly, LNCaP cells express an even higher and more uniform MscCa channel density than PC-3 cells (Figs. 5b, c) with a similar single channel conductance as seen in

PC-3 cells (Fig. 5d). However, whereas LNCaP cell channels are gated predominately in a transient mode (TM) in which channels close rapidly (i.e., in ≤ 100 ms) at the onset of a pressure pulse (117 out of 135 (87%) patches), the PC-3 cell channels show sustained gating (SM) and can remain open even after the pressure pulse (100 out of 118 (85%) patches) (compare Figs. 6a & 6b). These gating differences should have profound effects on the Ca^{2+} influx, which can be most clearly demonstrated by comparing responses to pressure steps and ramps (c.f., Figs. 6a & 6c with Figs. 6b & 6d). In LNCaP cells, a pressure step activates a large peak current of ~120 pA, whereas a ramp to the same pressure level activates a much smaller peak current of only ~ 2 pA (Fig. 6a & c). In contrast in the PC-3 cell patch shown in Figs 6b & 6d, steps and ramps activate similar amplitude sustained currents of ~40 pA. The MscCa activity induced by ramps and steps displayed the same reversal potential of ~ 0 mV and could be abolished with Gd^{3+} and GsMTx-4 irrespective of the stimuli waveform.

The TM gating that predominates in LNCaP cells arises though inactivation rather than adaptation since closed channels cannot be reopened by simply increasing the stimulus (Fig. 6a, see also 13, 15, 18). Instead, the stimulus must be turned off and reapplied, indicating the existence of an inactivated state that requires a finite time for recovery (Fig. 6b). Because the channel can enter the inactivated state from a closed state (i.e., without opening), this would account for why most channels are not available to be activated during the slower ramp stimulation. Unlike Na^{+} channel inactivation the pressure-dependent inactivation of MscCa is not strongly voltage dependent (Fig. 6c) but is subject to mechanical modulation (15, 42). For example, repetitive pressure pulses of 80 mmHg

of 1 s (but not 0.1 s) duration can result in irreversible loss of the transient current without increasing the sustained current. This response to over mechanical stimulation is similar to that reported for MscCa in other cells types and has been proposed to arise from decoupling of the membrane from the underlying CSK (15, 42). In PC-3 cells MscCa gating is more resistant to run down. As discussed below this cell-type difference in response to mechanical stimulation is consistent with the previously reported intrinsic difference in membrane-CSK decoupling and bleb formation seen between PC-3 and LNCaP cells (17).

$[Ca^{2+}]_i$ dynamics in LNCaP cells. We next asked whether the non-migrating LNCaP cells display $[Ca^{2+}]_i$ fluctuations, and if so, whether MscCa plays a role in shaping them. Of the 20 LNCaP cells studied in detail, none of the cells developed the sustained $[Ca^{2+}]_i$ gradient characteristic of migrating PC-3 cells. The lack of sustained $[Ca^{2+}]_i$ gradients is consistent with the uniform distribution of MscCa and ER/Ca^{2+} stores (Fig. 5) as well as the inability of MscCa to transduce stretch into sustained Ca^{2+} influx (Fig. 6). On the other hand, 16 out of 20 LNCaP cells did show repetitive $[Ca^{2+}]_i$ transients that spread throughout the cell (supplementary Fig. 4a). These transients were faster ($\leq$ 0.5 min) and occurred with a higher frequency (i.e., 8 $\pm$ 4.3 transients/h) than the transients observed in some PC-3 cells. Application of GsMTx-4 (3 μM) inhibited the Ca^{2+} transients/waves (supplementary Fig. 4b). The other cells that did not show Ca^{2+} transients displayed localized regions of elevated $[Ca^{2+}]_i$ that were associated with contractile or "tugging" activity at the end of the cell or with membrane protrusive "blebbing" activity (supplementary Fig. 4c). These local $[Ca^{2+}]_i$ elevations did not

develop in 10 out of 10 LNCaP cells that were incubated in 3 μM GsMTx-4 (data not shown).

DISCUSSION

Our results show that MscCa (also known as SAC) is expressed in the two most commonly studied human PC cell lines — the motile PC-3 line, originally isolated from a PC patient's bone metastasis, and highly invasive when implanted in nude mice (20, 48), and the nonmigratory LNCaP line, originally isolated from a patient's lymph node metastasis and noninvasive in nude mice (8, 19). We demonstrate that MscCa activity is essential for PC-3 cell migration based on reversible block produced by Gd^{3+} and GsMTx-4. Recently, it has been reported that GsMTx-4 also blocks directional migration in transformed MDCK-F cells (6). However, because MscCa is widely expressed in both motile and nonmotile cells (14, 36), it has not been clear what additional mechanisms, channel or otherwise, might be required to confer directional migration on non-motile cells. Our results indicate a specific mode of MscCa gating and consequent form of $[Ca^{2+}]_i$ dynamics, may be required for directed migration. In particular, because MscCa in PC-3 cells can remain fully open during stretch, the channel can support a sustained Ca^{2+} influx that is required to maintain a $[Ca^{2+}]_i$ gradient. This contrasts with the LNCaP cells in which the channel rapidly inactivates (< 100 ms) and therefore incompatible of the sustained Ca^{2+} influx required for any long term $[Ca^{2+}]$i gradient (1). We also find that the third major human PC cell line, the migratory DU-145 (isolated from a brain metastasis) expresses MscCa with a sustained gating mode (supplementary Fig. 5).

A key issue for MscCa is whether the extrinsic forces applied to activate the channel in the patch or the whole cell (25, 26) can also be generated intrinsically by the CSK. Clearly cells undergoing the mesenchymal form of migration become hyper-extended (i.e., up to 5 times their length), and this extension/spreading results in a visible smoothing out of membrane folds and microvilli (5). Furthermore, some migrating cells show elastic recoil as stretching forces exceed the strength of adhesions (27). Even more dramatic consequences occur when the retraction mechanisms are blocked and the stretching forces exceed the elastic limits of the bilayer, resulting in membrane rupture and cell fragmentation (45). Presumably, activation of MscCa and down-stream retraction mechanisms normally prevents this catastrophic event. Regarding the mechanism of MscCa gating it has been demonstrated that the force-generating CSK in migrating cells is equally linked to contacts within the dorsal and ventral matrix (10). In this case one would expect an isotropic increase in bilayer tension and activation of channels. However, while several lines of evidence support direct gating of MscCa by bilayer tension, similar to the various stretch-activated channels in bacteria (23, 29, 50), a recent elegant study carried out by Sokabe and colleagues has shown that direct tugging on actin stress fibers can cause local MscCa activation at the ventral cell surface (16). It still remains to be determined whether this localized channel activation occurs through direct actin-channel interactions or indirectly via increase in local bilayer tension, and whether similar local events are associated with cell migration.

Functionally, the $[Ca^{2+}]_i$ gradient seen in PC-3 cells would be expected to polarize the activity of Ca^{2+}-dependent molecules (e.g., transporters, enzymes, motors and adhesions) which in turn would coordinate polarization of the cell, and in particular promote the development of a single prominent and persistent lamellipodium essential for directional locomotion. On the other hand, the absence of this $[Ca^{2+}]$i gradient and Ca^{2+}-induced polarization in LNCaP cells may be permissive for the multiple mini-lamellipodia that arise transiently and randomly around the perimeter of the LNCaP cell. Although our results indicate that the MscCa-dependent $[Ca^{2+}]_i$ transients expressed by LNCaP cells are not sufficient to coordinate locomotion in this cell, they do play different roles in coordinating migration in other cell types (2, 3, 22, 25, 29, 28, 30). One view is that they activate proteins (e.g., calpain and/or mysosin II) that promote rear retraction (25), while another view is that they are more important in the development of traction forces at the cell front (30). In some of our PC-3 cell recordings we did see $[Ca^{2+}]_i$ transients that spread though out the cell, and on occasion they immediately preceded retraction (see supplementary video 4). However, their low frequency (or complete absence) in other PC-3 cells did not prevent cell migration, nor did their higher prevalence and frequency in LNCaP cells promote migration. It therefore seems that a sustained $[Ca^{2+}]_i$ gradient is key in determining directed migration in PC cells. This also seems to be the case for migrating cerebellum granule cells in which experimentally induced reversal of the $[Ca^{2+}]_i$ gradient is always accompanied by reversal in migration direction whereas the occasional occurrence of $[Ca^{2+}]_i$ transients cannot be causally related to directed migration (47). Most recently it has been reported (6) that in migrating Mardin-Darby kidney cells the highest $[Ca^{2+}]_i$ is seen at the very leading edge (see also 22), and this region of elevated

$[Ca^{2+}]_i$ appears to be superimposed on a less prominent $[Ca^{2+}]_i$ gradient that increases from the front to the rear of the cell. In this case, several forms of spatial $[Ca^{2+}]_i$ polarization may be involved in directing cell migration, which may vary cell type and/or mode of migration (4, 22, 28).

Our results also raise the possibility that a shift in Ca^{2+} dynamics mediated by changes in MscCa gating could switch PC cells from a nonmigratory to a migratory mode and vice versa. Indeed, previous studies indicate that MscCa can undergo a shift gating mode in response to mechanically-induced changes in interactions between the membrane and CSK. In particular, it has been shown that either mechanical over stimulation of the patch or membrane "blebbing" of the cell (15, 42, 50) can shift MscCa gating from the TM to the SM. In this case, it seems highly relevant that PC-3, but not LNCaP cells, exhibit spontaneous membrane blebbing, as well as "cytochalasin-induced" membrane blebbing on their apical surface (17). This apparent difference in the strength of membrane-CSK interactions may account for why SM gating predominates in PC-3 cells, and why mechanical over-stimulation of LNCaP cell patches, which itself promotes membrane blebbing, can switch gating from the transient to the sustained mode (15). However, a simple gating switch may not alone be sufficient to trigger directed migration since MscCa surface expression also differs between PC-3 and LNCaP cells, as presumably does the expression and/or coupling of the channel with downstream signaling pathways. Nevertheless, the idea of a mechanically-induced switch in cell motility is interesting given that during tumor progression, the cortical CSK does become more depolymerized (41) and Rho-induced membrane blebbing can lead to a switch in tumor cell motility

mode (37). Furthermore, it has been proposed that the elevated solid stresses and interstitial fluid pressures associated with a proliferating tumor, may actually promote increased tumor cell motility and cellular escape mechanisms from the tumor (32). At least consistent with this idea, is the demonstration that external mechanical forces can directly stimulate CSK polarization and persistent locomotion in cells and cell fragments (7, 45).

Clearly the identification of the protein subunits that form MscCa will be critical in more detailed understanding of its role in migration. However, at this time the exact protein composition of MscCa remains unresolved (11, 28, 40). So far two different members of the canonical transient receptor potential (TRPC) family, TRPC1 (28 & 29) and TRPC6 (40) have been proposed to form MscCa. However, there is also conflicting evidence on whether either subunit w expressed alone can form a directly gated MS channel (11). On the other hand, a recent report published online indicates that TRPC1 suppression blocks and TRPC1 overexpression stimulates migration (6). This compares with our observations indicating that either TRPC1 suppression or overexpression blocks PC-3 cell migration, whereas TRPC6 suppression is without effect (R. Maroto, A. Kurosky & Hamill, unpublished observations). Studies to resolve these discrepancies and determine the effects on functional expression of MscCa and other channels are currently underway.

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Figure legends

Figure 1. MscCa properties in PC-3 cells. **a:** Left panel is a photomicrograph indicating three distinct morphological regions: the front region that includes the lamellipodium and lamella (L), the cell body (B), and the rear tether (R). The right panels show cell-attached patch recordings made from each region on different PC-3 cells. Over all regions ~15% of patches were null for stretch-activated currents with a decreasing % of null patches towards the cell rear (L = 24%:10 out 42 patches; B= 15%: 8 out 54; T= 11%: 3 out 28). The mean current amplitudes (at -50 mV) excluding null patches were L= 23.5 ± 3.58 pA (n = 22); B = 24.5 ± 2.56 pA (n = 38); and R = 26.0 ± 3.8173 (n = 22). **b:** Suction step applied to a cell-attached patch activated single channel currents measured at -50 mV and 50 mV. Note the more frequent fast closures and reopenings at the negative potential. **c:** Single-channel current-voltage relations measured on cell-attached PC-3 patches (solid symbols) and *Xenopus* oocyte patches (hollow symbols) with zero Ca^{2+} (circles, pipette solution in mM: 100 KCl (or 100 NaCl), 5 Hepes, 2 EGTA) and 1 mM Ca^{2+} (triangles, pipette solution in mM 100 NaCl (or 100 KCl), 5 Hepes 1, $CaCl_2$). In the various pipette solutions the chord conductance at -50 mV was 55 pS (100 K^+:0 Ca^{2+}), 42 pS (100 Na^+:0 Ca^{2+}) 25 pS (100 K^+:1 Ca^{2+}) and 20 pS (100 Na^+: 1 Ca^{2+}) based on 4-10 patches for each

cell type and each ionic condition. **d:** Consecutive current traces showing that pressure activation of inward current results in delayed activation of unitary outward channel currents of ~1 pA (pipette solution; 100 NaCl, 5 Hepes and 1 mM $CaCl_2$, patch potential: ~ -10 mV). The same delayed activation of outward currents was seen in 5 other patches but was absent in 3 patches recorded with Ca^{2+} removed from the pipette solution. Ca^{2+} influx via the stretch-activated MscCa is able to selectively activate the intermediate conductance (~ 20 pS) Ca^{2+}-activated K^+ (IK) channel without activating a larger (~200 pS) conductance $Ca2^+$-activated K^+ (BK) channel. The BK channels could be activated in all PC-3 cell patches so far studied by either strong depolarization (i.e., more positive than 20 mV) or by inclusion of 10 μM A238187 in the bath solution, which also activated the IK channels (Maroto & Hamill, unpublished observations). We take this selective activation to indicate that IK and MscCa channels are in close proximity and form a functional unit. BK channels may be located further apart from MscCa and/or require higher $[Ca^{2+}]_i$ elevations possibly involving CICR from Ca^{2+} stores that are displaced during tight seal formation. The selective coupling between MscCa and IK channels was also seen in patches formed on LNCaP cells (Maroto & Hamill, unpublished observations).

Figure 2. PC-3 cell migration and the effects of Gd^{3+} and GsmTx-4. **a:** Selected video frames 30 min apart showing PC3 cells migrating out of a cluster. **b:** Representative trajectories (monitored every 5 minutes) before, during, and after application of 5 μM Gd^{3+} and 3 μM GsMTx-4. **c:** Histograms based on 25 or more cells (mean ± SEM) showing reversible block of migration by Gd^{3+} and GsMTx-4.

Figure 3. Intracellular Ca^{2+} gradients and transients in migrating PC-3 cells. **a: [**$Ca^{2+}]_i$ fluorescent images from left to right of a PC-3 showing regions of high $[Ca^{2+}]_i$ that develop initially in the rear half of the cell and spread later to the front of the cell. This particular cell was monitored for 3.5 h and shown in supplementary video 2. **b:** $[Ca^{2+}]_i$ images of two PC-3 cell initially migrating in opposite directions and with opposite $[Ca^{2+}]_i$ gradients (30 min frame) (arrows). In the next frame (125 min) cell 1 had reversed its $[Ca^{2+}]_i$ gradient and migration direction. The cell then showed a Ca^{2+} transient (136 min frame) that was followed by retraction of its tether (145 min) and continued cell movement (186 min). The original recording was made over 4.8 h and is shown in supplementary video 4. **c:** Fast Ca^{2+} transients in a migrating PC-3 cell. Images from left to right show two PC-3 cells, in which the migrating cell (#1) undergoes $[Ca^{2+}]_i$ transients while the stationary cell (#2) does not (see also supplementary video 5). For all images a 100X 1.3 NA objective was used. **d:** GsMTx-4 reversibly reduced $[Ca^{2+}]_i$ elevations in PC-3 cells. Three $[Ca^{2+}]_i$ images showing PC-3 cells before, after 5 min exposure to 3 μM GsmTx-4 solution and 30 minutes following GsmTx-4 washout in which the $[Ca^{2+}]_i$ had overshot levels before GsMTx-4 exposure. These images were taken with a 20X 0.75 objective. The ratiometric fura-2 measurements shown here permits the monitoring of free $[Ca^{2+}]_i$ independent of uneven dye distribution due to changes in cell thickness and/or dye accumulation in organelles. In particular, the apparent low $[Ca^{2+}]$ seen in the PC-3 cell nucleus ($[Ca^{2+}]_n$) compared with the cytosol ($[Ca^{2+}]_c$) is not due to low nuclear dye because the raw fluorescence images excited by 340 and 380 nm actually showed higher nuclear fluorescence indicating higher dye presence. Moreover the nucleus has a

relatively large free volume for fura-2 to occupy. However, the ratiometric measurements, by correcting for any "false" fluorescence due to the thickness and dye accumulation, indicates a relatively low free $[Ca^{2+}]_n$. Although there has been debate on the significance of $[Ca^{2+}]_{n/c}$ gradients, and it is possible that the nuclear environment changes dye properties, previous studies have reported the same or even opposite N/C gradients in other cell types.

Figure 4. The distribution of ER and mitochondria in PC-3 cells. **a & b:** Transmission and immunofluorescent confocal images of a PC-3 cell labeled with 200nM BODIPY FL-thapsigargin indicates a higher distribution of the ER/internal Ca^{2+} stores within the cell body compared with the lamella and lamellipodium. The images represent the maximum intensity projection reconstructed from a stack of 25 confocal sections obtained at 0.2 µm intervals. **c**. Confocal image of a PC3 cell loaded with Mitotracker-Red (100 nM) to visualize mitochondria distribution. The fluorescent image represents the maximum intensity projection reconstructed from a stack of 20 confocal sections obtained at 0.2 µm interval using a 63X1.4 objective (excitation 581, emission 644). DAPI (emission 457) was simultaneously applied to visualize the nuclei. 14 out of 16 cells showed a similar pattern.

Figure 5. The non-migratory LNCaP cell also expresses MscCa. **a**. Photomicrogaphs showing transmission and confocal fluorescent images of an LNCaP cell labeled with BODIPY FL-thapsigargin to visualize ER distribution. The fluorescent image represents

the maximum intensity projection reconstructed from a stack of 30 confocal sections obtained at 0.2 μm intervals. Both transmission and fluorescent images are overlapped indicating an almost uniform ER distribution within the cytoplasm. **b.** Current response of a cell-attached patch from a LNCaP cell to a 60 mmHg suction step which showed a peak current of approximately 140 pA indicating ~70 channels in the patch that inactivated within 100 ms of the step. **c**: Histogram showing that LNCaP cells express relatively larger peak currents in response to pressure steps (based on 118 patches) compared with responses of patches on PC-3 cells (based on 135 patches). **d**: Single channel current-voltage relations measured for LNCaP and PC3 cells (with 100 mM KCl 2 EGTA (KOH) and 5 mM Hepes (KOH) in the pipette solution) superimpose indicating the same or closely-related pore structure. Data points based on 10-20 patches for each PC cell type.

Figure 6. Comparison of LNCaP and PC-3 cell responses to pressure steps versus ramps. **a:** Responses of the same membrane patch on an LNCaP cell to increasing pressure steps and ramps. The largest step of 100 mmHg activated a peak current of ~130 pA, compared with only ~2 pA for the ramp up to 100 mmHg. Similar discrepant responses were seen whether ramps were applied before or after steps. **b:** Similar protocols as in a applied to a PC-3 cell membrane patch in which both increasing steps and ramps produced similar maximal sustained currents of ~ 40 pA. **c:** Expanded records of ramp responses on a different LNCaP cell patch. At the very beginning of the ramp currents representing 2 channels were briefly activated but then inactivated with increasing ramp pressure. Towards the peak of the ramp pressure additional noisy currents were seen and both types

of currents were equally blocked by Gd^{3+} and GsMTx-4 (data not shown). The noisy currents may represent the inactivated channels reopening as lower sub-conductance states as has been reported for the inactivated MscCa in astrocytes (42). **d:** Expanded ramp responses on another PC-3 cell patch showing the sustained opening during the ramp and delayed closing after the ramp. Although the experimentally applied ramps here are clearly not as long as that expected to develop during the cell during migratory cycle which can last several hours, the absence of MscCa inactivation would allow for sustained Ca^{2+} influx over this time scale.

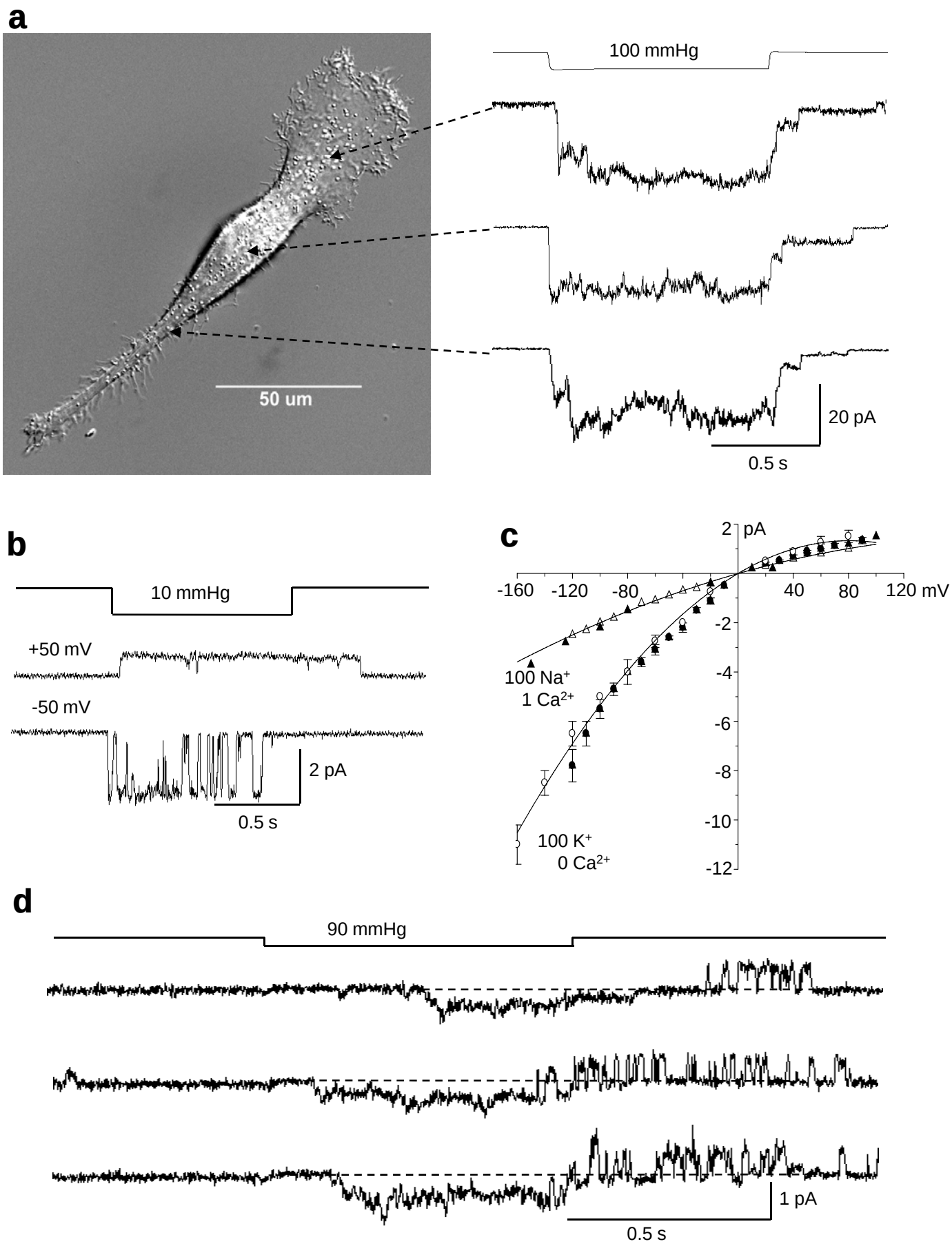
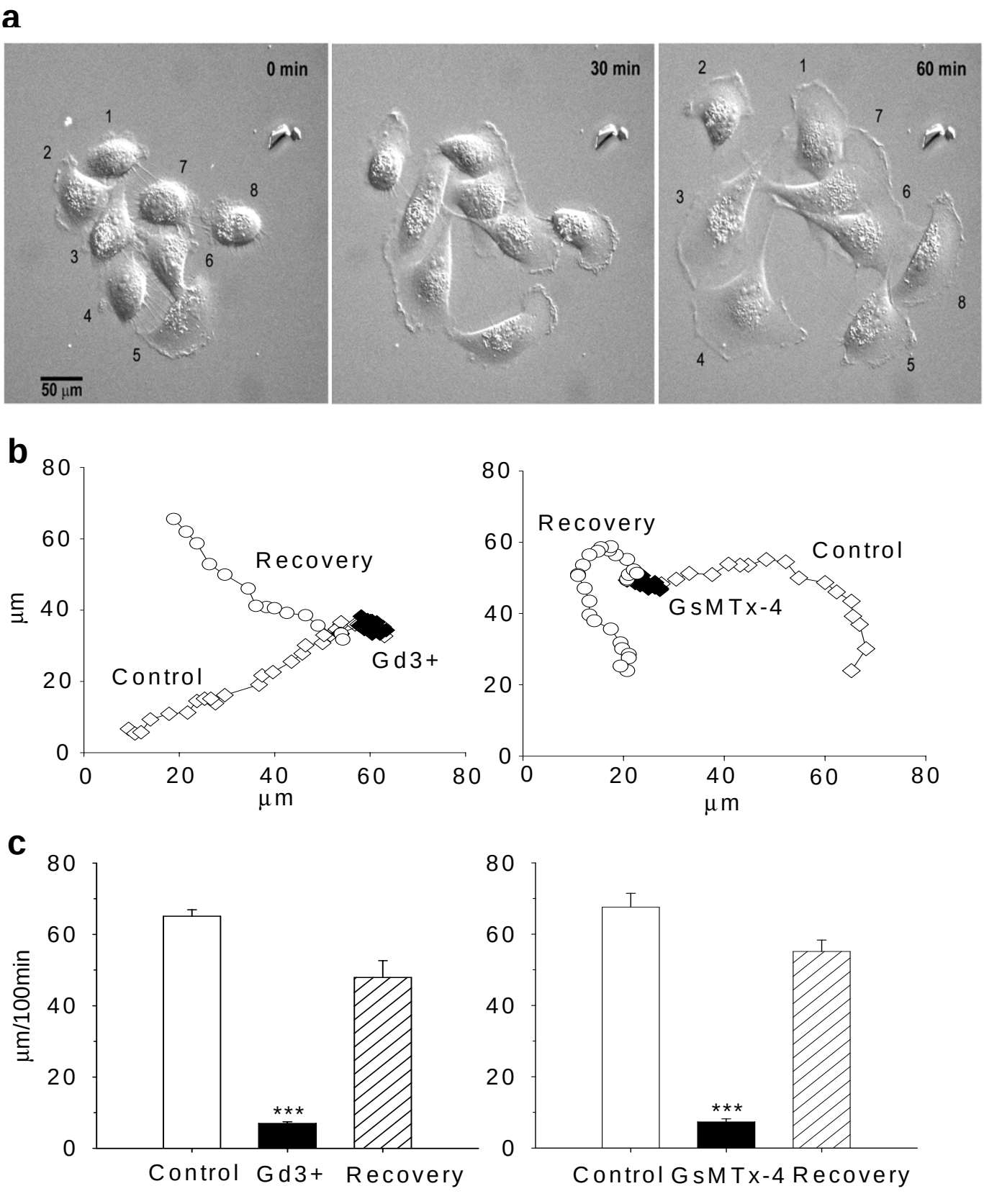

a
100 mmHg
50 um
20 pA
0.5 s
b
10 mmHg
+50 mV
-50 mV
2 pA
0.5 s
c
2
pA
-160
-120
-80
40
80
120 mV
-2
-4
-6
-8
-10
-12
100 Na+
1 Ca2+
100 K+
0 Ca2+
d
90 mmHg
1 pA
0.5 s

Fig 1


a
0 min
30 min
60 min
50 μm
b
Recovery
Control
Gd3+
μm
Recovery
Control
GsMTx-4
μm
c
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Gd3+
Recovery
***
Control
GsMTx-4
Recovery

Fig 2

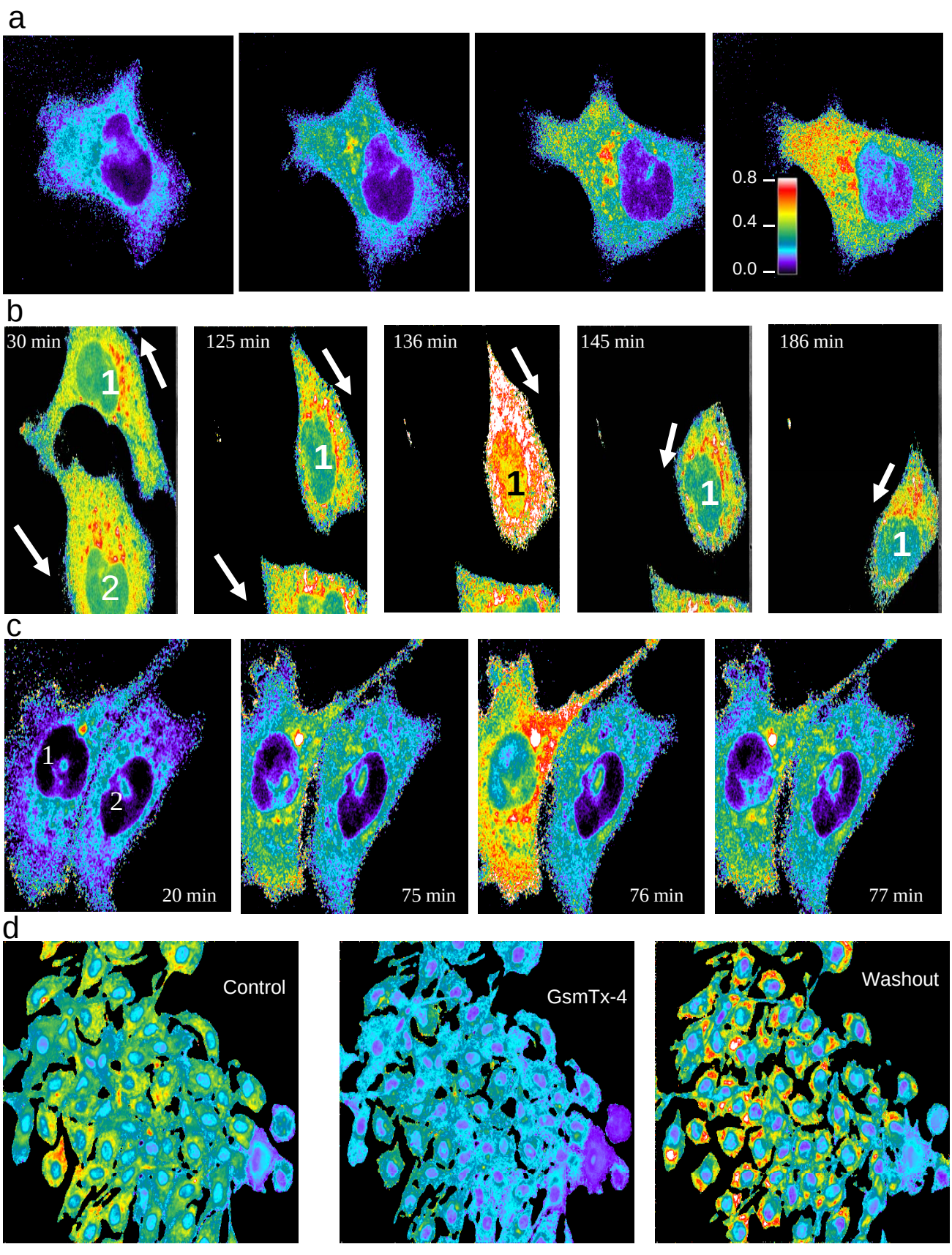

a
0.8
0.4
0.0
b
30 min
125 min
136 min
145 min
186 min
1
2
c
20 min
75 min
76 min
77 min
d
Control
GsmTx-4
Washout

Fig 3

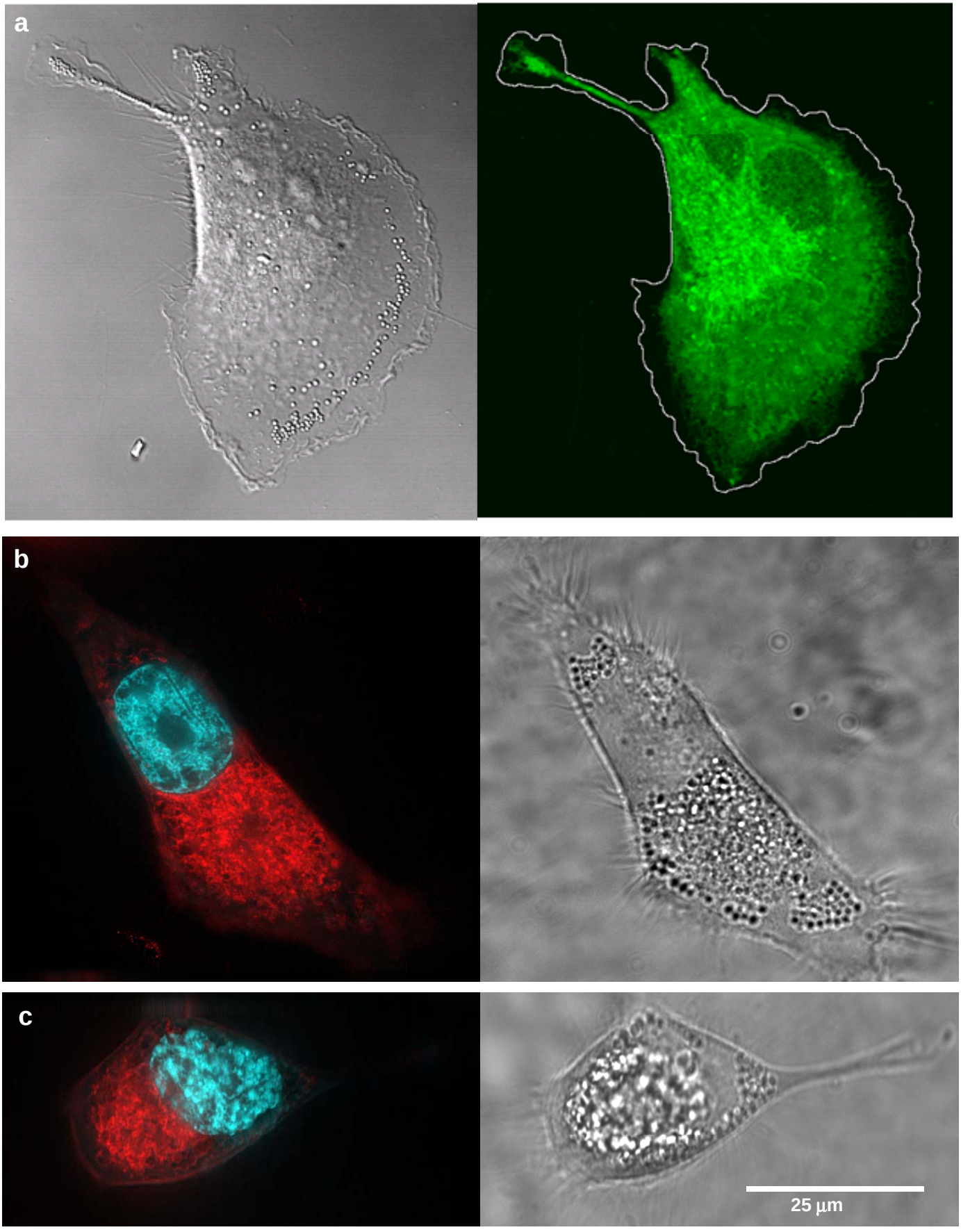
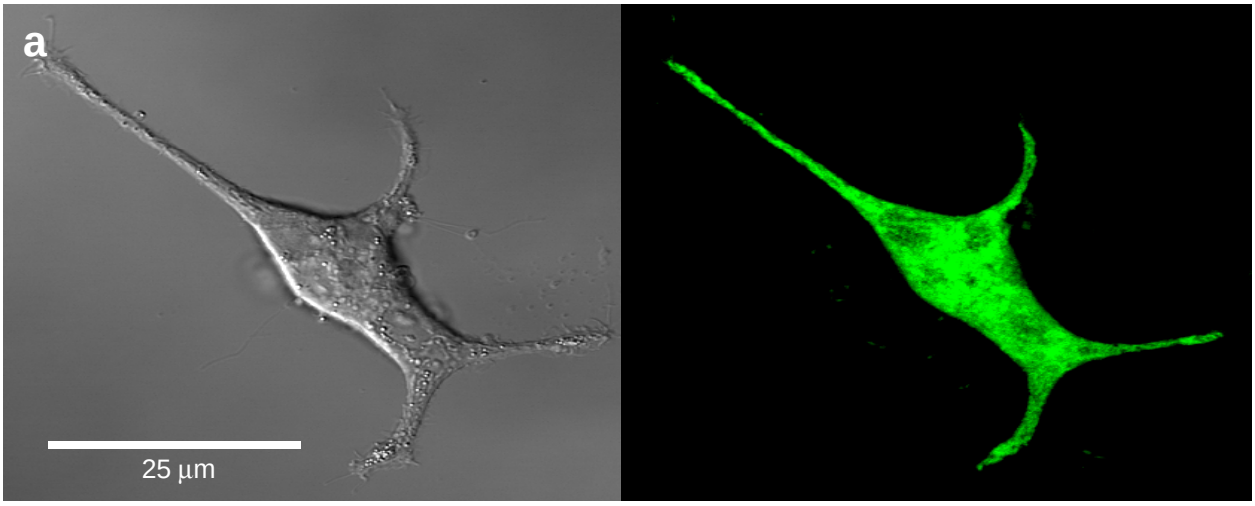
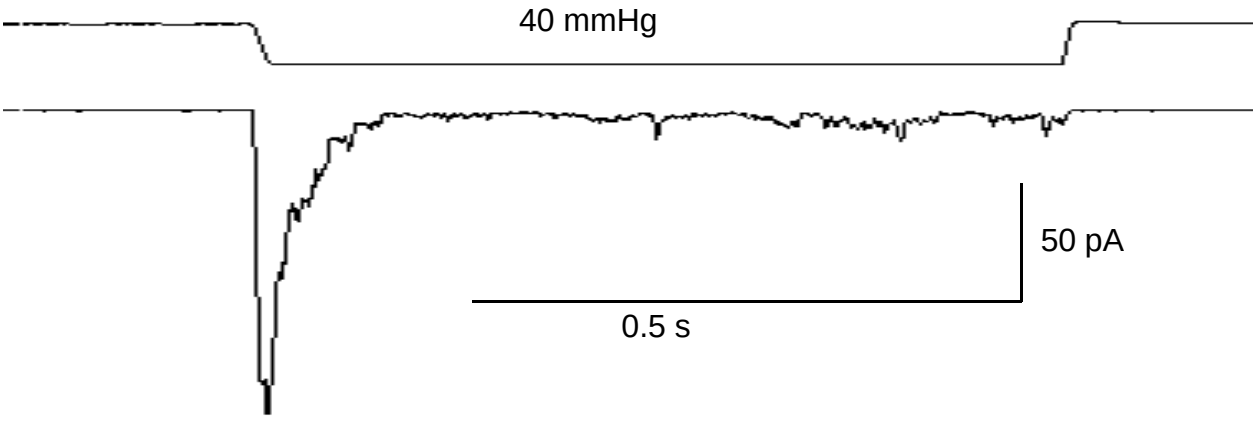
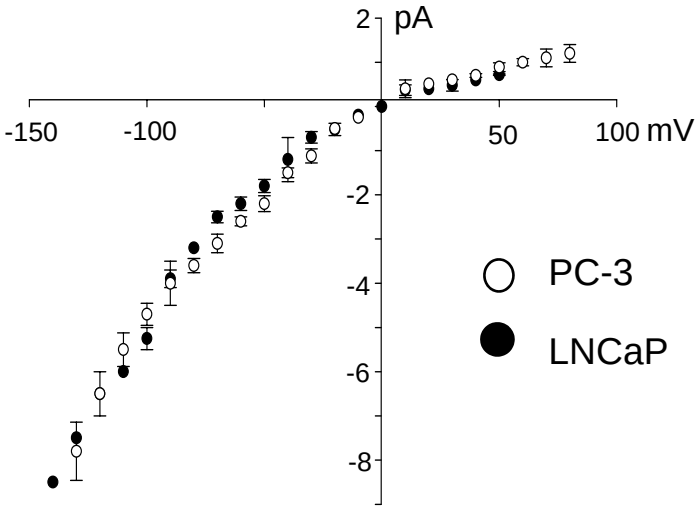


Fig 4


a
25 µm


40 mmHg
50 pA
0.5 s


pA
2
-2
-4
-6
-8
-150
-100
50
100 mV
PC-3
LNCaP

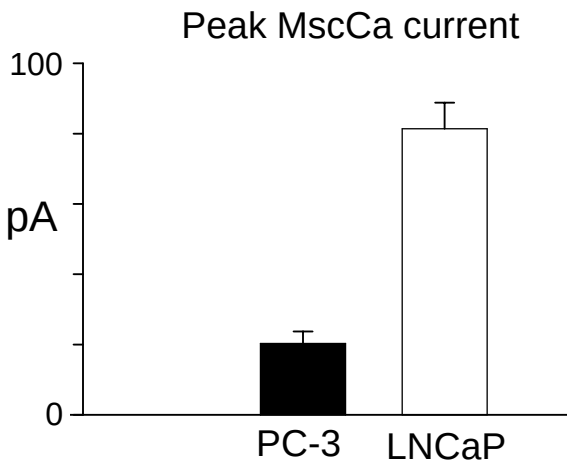
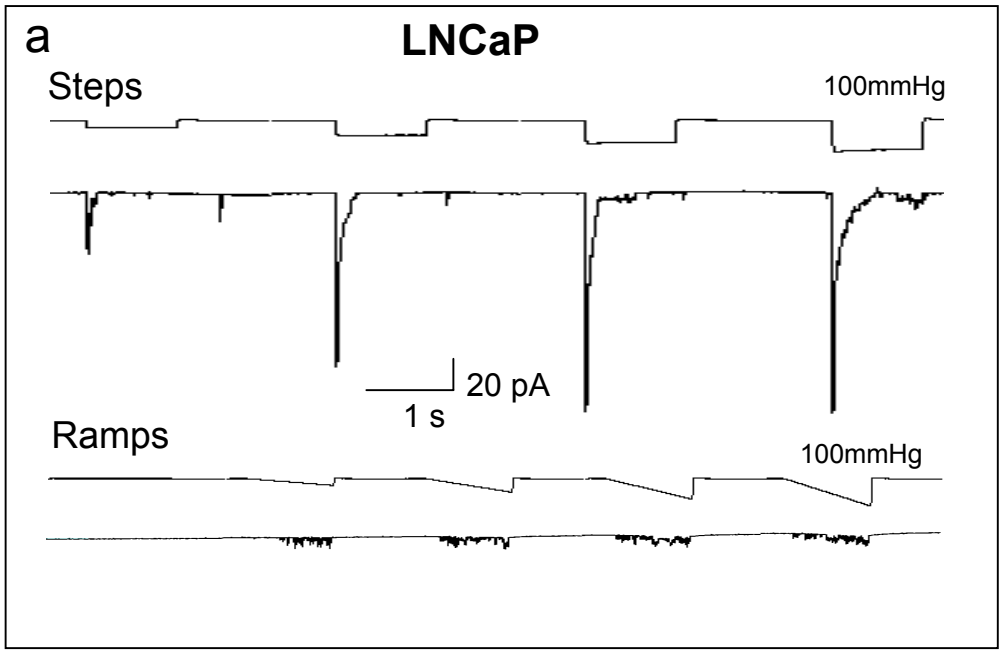
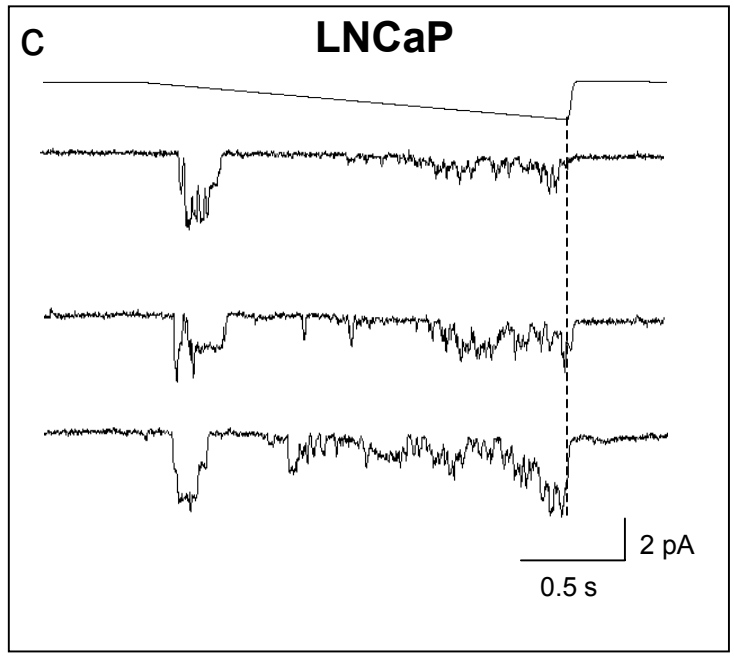

Peak MscCa current
100
pA
0
PC-3
LNCaP

Fig 5


a
LNCaP
Steps
100mmHg
20 pA
1 s
Ramps
100mmHg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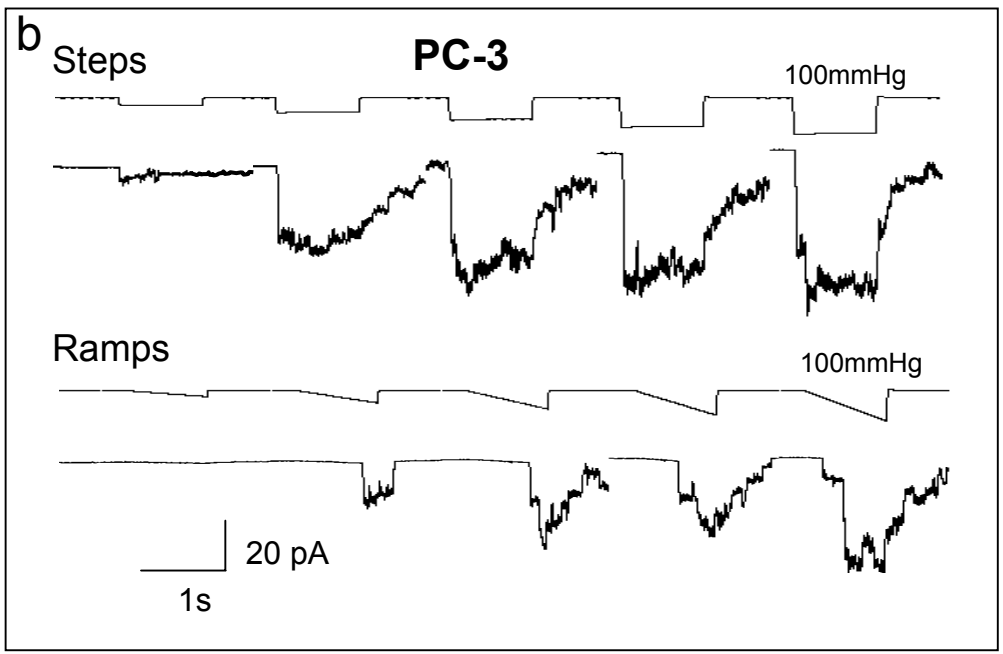
2 pA
0.5 s


b
PC-3
Steps
100mmHg
Ramps
100mmHg
20 pA
1s

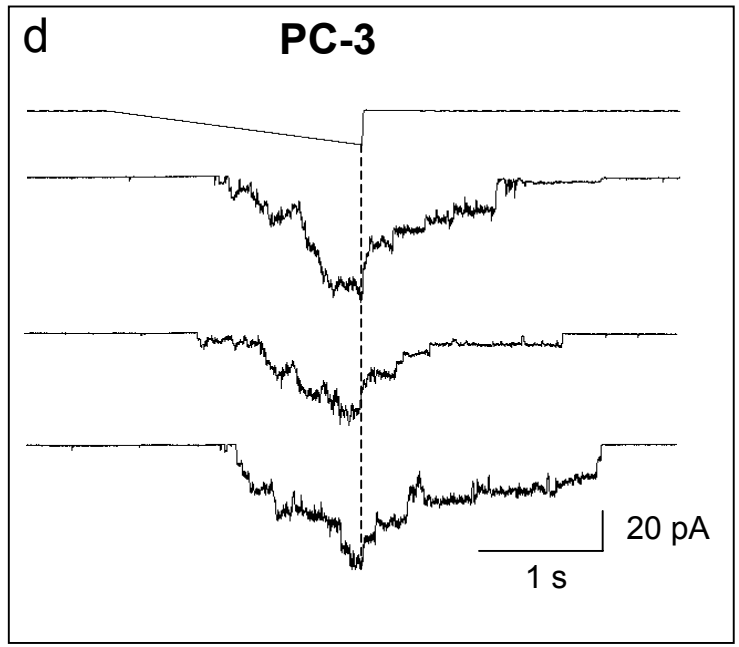

d
PC-3
20 pA
1 s

Fig 6

Stretch-activated calcium channel activity is required for human prostate tumor cell migration

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In its early stages prostate cancer (PC) stays in the prostate and is not life-threatening, but without treatment it spreads and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade, it is important to identify the mechanisms that regulate PC cell migration. In order for any cell to migrate there must be coordination between its forward protrusion and rear retraction. It has been hypothesized that the mechanosensitive stretch-activated Ca^{2+}-permeable channel (MscCa) plays a central role in this coordination by providing feedback between cell extension and the Ca^{2+}-sensitive proteins (e.g., myosin II and calpain) that promote cell contraction and/or rear adhesion disassembly. With the funding support provided by a 2004/5 Exploration Hypothesis Development Award we tested this hypothesis by using patch-clamp recording to measure whether MscCa is expressed in the highly motile/invasive human prostate tumor cell line PC-3, and if so, how this channel activity might influence Ca^{2+} concentration ($[Ca^{2+}]_i$) changes associated with cell migration measured using intracellular $[Ca^{2+}]_i$ imaging and time-lapse video microscopy. At the same time we also examined MscCa activity and $[Ca^{2+}]_i$ changes in the non-motile/noninvasive PC cell line LNCaP. Our results indicate that MscCa is expressed in PC-3 cells and is essential for PC-3 cell migration. In particular, we found that several agents including the lanthanide Gd^{3+}, a nonspecific MscCa blocker, and the tarantula venom peptide, GsMTx-4, a specific MscCa blocker, inhibited $[Ca^{2+}]_i$ transients and gradients and also blocked tumor cell migration. These results support our original hypothesis that MscCa activity is required for PC cell migration. However, MscCa activity is not sufficient for PC cell migration, since MscCa is expressed at higher membrane patch current density in LNCaP compared with PC-3 cells. Furthermore, although LNCaP cells do not develop the sustained intracellular $[Ca^{2+}]_i$ gradient (increasing from front to rear) that determines PC-3 cell directional migration, LNCaP cells do express robust fast $[Ca^{2+}]_i$ transients associated with random membrane protrusive activity, indicating that it is a polarized and sustained activation of MscCa that is critical for tumor cell migration.

IMPACT STATEMENT: There is now urgent need to identify novel and susceptible molecular mechanisms that may be targeted to block PC invasion and metastasis. Our studies indicate that MscCa and the physical/biochemical mechanisms that regulate its polarized activation should be promising targets to block PC cell migration and invasion.

Expression and function of canonical transient receptor potential channels in human prostate tumor cells

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Pharmacological inhibition of the mechanosensitive stretch-activated calcium channel (MscCa) blocks human prostate cell migration, a critical process in the metastatic cascade (Hamill & Maroto, IMPACT meeting 2011). However, in order to target this channel with molecular and genetic techniques it is crucial to identify the specific proteins that form MscCa. One candidate protein family known to form Ca^{2+} permeable cation channels is the canonical transient receptor potential (TRPC) channel family, which consists of seven members subdivided into the two subfamilies, TRPC1, 4 & 5 and TRPC3, 6 & 7, (TRPC2 is a pseudo gene in humans and is not expressed). In particular, separate studies have implicated TRPC1, TRPC5 and TRPC6 in forming MscCa. With funding support provided by a 2006 New Idea Award we used immunoblot techniques to measure TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 expression in human prostate tumor cells lines. Our results indicate higher expression of TRPC1 and TRPC3 in the non-migratory LNCaP cell line compared with the migratory PC3 cell line — this expression pattern is consistent with the higher MscCa patch channel density in LNCaP versus PC-3 cells. TRPC6 was weakly but equally expressed in LNCaP and PC-3 cells. In contrast, TRPC4 was not detected in PC-3 cells although present in LNCaP cells, and TRPC5 could not be detected in either tumor cell line. Selective suppression of TRPC1 or TRPC3 but not TRPC6 using small interfering RNA (siRNA) blocked PC3 cell migration as measured by time lapse video microscopy and wound closure. Overexpression of TRPC1 but not TRPC3 also blocked PC-3 cell migration. Patch clamp recordings from cells in which either TRPC1 or TRPC3 was suppressed indicated that MscCa activity was not significantly reduced compared with scrambled controls. Furthermore, overexpressing TRPC1 or TRPC3 did not increase MscCa activity. In conclusion, TRPCs regulate PC cell migration but do not appear to directly form MscCa.

IMPACT STATEMENT: It is important to identify novel and susceptible molecular targets to block PC invasion and metastasis. Our studies indicate that genetic suppression of TRPC1 and TRPC3 are effective in abolishing PC cell migration and therefore provide new avenues for blocking prostate tumor invasion.